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THE ANALYSIS OF BIOLOGICAL FLUIDS FOR ACYLCARNITINES

A thesis submitted for the Degree of Doctor of Philosophy

by

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B.Sc. Hons. (Central Lancashire University, 1990)

March 1999

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The Open University

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DEDICATION

To Frank

ACKNOWLEDGEMENTS

Thanks to my family without their backing this may not have come to be and to Carl my constant companion and inspiration when completion seemed a long way off.

I wish to thank Dr Malcolm Rose for his help, guidance and friendship through both the practical part of this thesis work at The Open University and in the long haul since then, thank you for not loosing faith. Also to Barbara Rose for opening her door to me during my first homeless days in MK. Thanks to Dr Jim Iley and Dr David Roberts for taking up the gauntlet and to the rest of the Dept. of Chemistry staff for their friendship and support.

Thanks also to the Foundation for the Study of Infant Death (FSID) for funding this project and to The Institute of Child Health and Queen Elizabeth Hospital (London), Temple Street Children's Hospital (Dublin) and Dr P Divry (Lyon) for providing clinical samples for analysis.

ABSTRACT

This thesis outlines the development and evolution of a method for the analysis of biological fluids for acylcarnitines. These compounds are of interest due to their role in the metabolism of fatty acids and their use as indicators of metabolic defects, which have been associated with SIDS (Sudden Infant Death Syndrome).

These compounds are zwitterionic and thermally labile in nature and would not traditionally lend themselves to gas chromatographic – mass spectrometric (GC-MS) analysis. Described in this thesis is a method whereby lactonisation of the acylcarnitine facilitates the volatility required for GC analysis while retaining the side chain identifier to the original acylcarnitine for mass spectrometric identification. Using this method it has been possible to characterize acylcarnitines which are metabolic indicators, distinguish isomeric acylcarnitines chromatographically and show diagnosis of a number of diseases from clinical samples.

Sample analysis has been demonstrated for both blood and urine. In the case of urine samples an example has been provided to illustrate the advances in analytical techniques available for these analyses, to show the stability of these compounds in the matrix and the wealth of diagnostic information which can be obtained for an individual sample. The development of a method for the analysis of acylcarnitines from blood spots has been detailed. The application of this methodology to a number of disorders of fatty acid metabolism has been illustrated. The extension of the technique to the analysis of dicarboxylic acylcarnitines has also been investigated.

Finally with advances in modern technology the area of electrospray mass spectrometry has been evaluated for the analysis of samples without lactonisation. Results from this investigation are presented.

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LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
BIPM	<i>N</i> -[<i>p</i> -(2-benzimidazolyl) phenyl] maleimide
BSTFA	Bistrifluoroacetic acid
CAT	Carnitine acetyl transferase
CI	Chemical Ionisation
cm	centimetres
cm/s	centimetres per second
CPT(I and II)	Carnitine Palmitoyl transferase enzymes I and II
DCA	Dicarboxylic Acids
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EI	Electron Ionisation
ESI (ES)	Electrospray
ETF	Electron transfer Flavoproteins
ETF-DH	Electron transfer flavoprotein ubiquinone: oxido-reductase
FAB	Fast Atom Bombardment
FAD	Flavin adenine dinucleotide
FID	Flame Ionisation detection
GA	Glutaric acidemia
GC	Gas Chromatography
GER	Gastro-oesophageal reflux
HMG	Hydroxymethylglutaryl
HPLC	High performance liquid chromatography
ID	Internal diameter
IR	infrared
ITD	Ion trap detector

kV	kilovolts
l	litre
LC	Liquid Chromatography
LCAD(D)	Long-chain acyl-CoA-dehydrogenase (deficiency)
M	concentration (Molar)
m	metres
<i>m/z</i>	Mass to charge ratio
mA	milliamps
MCADD(D)	Medium-Chain Acyl-CoA Dehydrogenase (Deficiency)
mg	milligrams
min	minute
ml	millilitres
mM (mMol)	millimolar
mm	millimetres
MS	Mass Spectrometry
MS/ MS	Tandem Mass Spectrometry
NAD	Nicotinamide adenine dinucleotide
ng	nanograms
NMR	Nuclear Magnetic Resonance
¹³ C NMR	Carbon-13 NMR
¹ H NMR	Proton NMR
°C	degrees Celsius
Pa	Pascal
REA	Radioenzymic exchange assay
SEM	Scanning Electron Microscopy
SIDS	Sudden Infant Death Syndrome
SUDS	Sudden Unexpected Death Syndrome

TCA	Tricarboxylic acid
TLC	Thin layer chromatography
TML	ϵ -N-trimethyllysine
TMS	Trimethylsilane
UV	ultraviolet
V	Volts
μ A	microamps
μ g	micrograms
μ l	microlitres
μ m	microns

PUBLICATIONS ARISING FROM THIS THESIS

The following papers have been published or are in preparation and reflect work carried out towards this Ph.D. thesis.

B.M. Kelly, M.E. Rose, and S. Lowes, *in preparation*.

B.M. Kelly, M.E. Rose, D. Wycherley and S.W. Preece *Organic Mass Spectrom* **27**, 924-926 (1992).

The latter describes the initial electrospray work, which is described in Chapter 6 of this thesis.

CHAPTER 1

INTRODUCTION.

"The sudden death of an infant or young child, which is unexpected by history, and which a thorough post-mortem examination fails to demonstrate an adequate cause of death." J.P.Beckwith (1973) [1].

The quotation above is the accepted working definition of sudden infant death syndrome (SIDS) and was agreed at the Second International Conference on causes of Sudden Death in Infants held in Seattle in 1969. SIDS, colloquially known as "cot death", was found to occur with varying intensities within different communities but the accepted figure was of one to four for every thousand live births [2] and is the single largest cause of death in infants from one week to one year in age.

Evidence from epidemiological studies of SIDS demonstrates a higher occurrence in lower socio-economic groups with a higher incidence in the winter months. SIDS has also become more associated with premature infants, those of low birth-weights and those born to single mothers. Regarding the mother the risk can be increased in the case of smokers, opiate/barbiturate users, those suffering from infections during pregnancy and those with high parity and short inter-pregnancy intervals [2, 3, 4]. It has been suggested that SIDS, rather than a single disorder, is the end point of a complex group of inter-related factors [5, 6, 7] and there has been a number of distinct disorders linked with it.

Factors linked with SIDS include respiratory disorders, as in the case of the apnoea hypothesis [8]. These are cases where there is an abnormal disturbance in the breathing pattern, which, in the case of SIDS patients, is fatal. Though some studies seemed to provide evidence for this theory [6, 9], contradictory evidence has also been presented which leads to unclear definitions of normal breathing patterns [10, 11]. Most SIDS

victims are not known to have suffered apnoeic episodes, although evidence suggests that defects in the respiratory mechanism are responsible for a number of cases of sudden and unexpected deaths in infants. Further studies are required to determine the percentage of SIDS infants dying of respiratory causes and to elucidate the underlying defect [2].

Neuropathological hypotheses have also been proposed and these are defined as brain-stem dysfunction during sleep leading to cardiorespiratory instability [7]. The incidence of SIDS peaks around the first 2 - 4 months of life and these hypotheses relate this age group with changes in the neural control of respiratory and cardiac functions and the wake/sleep patterns of infants [5]. As in the previous apnoeic hypothesis there has been a range of conflicting neurological findings [5, 6]. Research has also linked neuropathological disorders to apnoea through chemoreceptor dysfunction, though again there are conflicting ideas on this link [5, 6, 12, 13] with the latter two groups [12, 13] proposing carotid body defects or changes as a possible cause of SIDS.

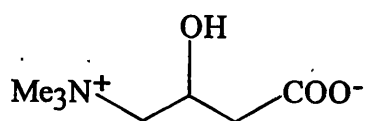
Debate has also taken place in the literature as to whether a factor known as a gastro-oesophageal reflux (GER) has a role in SIDS. In these cases the acidic stomach contents are expelled into the oesophagus instigating an apnoeic episode [14, 15]. Results in this area have not proved reproducible though there does seem to be a link with SIDS [14, 15, and 16]. Other proposals for the mechanism of SIDS include inherited disorders of the cardiac conducting system with death due to arrhythmia [17], infections from a wide range of sources including bacterial toxins [18, 19], and varied immunological disorders at local and general levels [20]. Sleeping position, with a lower incidence of SIDS when a babies sleep in a supine position [21], and avoidance of smoky atmosphere and overheating for infants have also been advised recently. This advice has led to a considerable reduction in the incidence of SIDS among the population of a number of countries (typically quoted at

about 50%). However, the reduction has not been universal [22] but there is little doubt that advice on sleeping position and wrapping has had a marked beneficial impact.

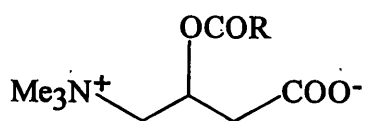
There has been a great deal of speculation in UK television programmes about the potential role of toxic gases emanating from soiled mattresses. However, three substantial review bodies have failed to demonstrate a definite link between cot death and the condition and type of mattresses. Most experts agree that the current largest risk factor concerns smoking (during pregnancy and in the baby's presence).

Inborn errors of metabolism have also been proposed as a factor in SIDS though, as with some of the other examples above, these can be identified at the stage of post-mortem in many cases and so the term Sudden Unexpected Death (SUDS) has been adopted to cover these deaths. Steatosis is recognised as a finding that occurs in a percentage of infants who die of SIDS/SUDS and though this is suggestive of an underlying metabolic disorder it is only in recent years that specific biochemical defects have been identified. A clue to the role of these disorders in the death of an infant is the identification of a fatty liver at post-mortem [23]. Some of these disorders can be assigned through the identification of metabolites (e.g. organic acids) in biological fluids and it is this area of study which is investigated in this presentation. One particular disorder associated with SUDS is known as Medium-chain Acyl-CoA Dehydrogenase Deficiency (MCADD) and results from a build-up of medium-chain fatty acid metabolites which have a toxic effect. Metabolism identification has proved vital in determining the pathways involved in this defect. This, along with a number of other metabolic disorders of fatty acid metabolism, will be discussed at length in this presentation.

This thesis will outline the development of analytical methods for the identification of trace metabolites of fatty acid oxidation, acylcarnitines, from urine and dried blood



1



2

Figure 1.1 Structures of Carnitine **1** and its *O*-acyl esters **2**

samples as a means of diagnosing infants at risk from SUDS. Before describing the method development it is necessary to have an outline of the history and metabolic role of this group of compounds and also to investigate other methods available for the analysis of carnitine and its *O*-acyl esters from a variety of sources.

Carnitine, **1**, (3-hydroxy-4-aminobutyrobetaine) and its *O*-acyl esters, **2**, (illustrated in Figure 1.1, opposite) are key substances in the metabolism of fatty acids. Their detection in biological fluids can be used for the diagnosis of a number of metabolic disorders. It is this latter feature which has in recent years led to a rise in interest in detection and characterization of carnitine and its esters, using a wide variety of methods and instrumentation. The aim of many of these investigations is to determine the presence of abnormal metabolites in the biological fluids, which may be indicative of specific enzyme defects.

1.1 HISTORY AND BIOSYNTHESIS OF CARNITINE.

In 1904 Franz Knoop first proposed that the metabolism of fatty acids involved oxidation at the β -carbon position. This early work concentrated on the use of chemical labelling to trace metabolic pathways. However, it was not until the 1950s, with the discovery of co-enzyme A, the isolation of fatty acids and the elucidation of the mechanisms involved that Knoop's work was confirmed [24]. Much of the mechanism of carnitine's interactions within the β -oxidation pathway is now understood. In 1905 [25], the empirical formula ($C_7H_{15}NO_3$) was assigned to a compound discovered in meat extract. It took a further twenty-two years for the structural formula to be proven as L-3-hydroxy-4-*N*-trimethylaminobutanoic acid [26]. The later discovery of carnitine in insects was of great interest as previously carnitine had only been identified from vertebrate muscle. In 1951 Carter *et al.* [27] established carnitine as Vitamin B_T with the first assay being carried out

on the mealworm, *Tenebrio molitor*, as Vitamin B_T was considered essential for its growth. Their assay for the presence of carnitine, The Tenebrio Test, was then applied to a range of biological materials.

In 1953 Fraenkel [28] was the instigator of the first carnitine assay applied to human urine and blood. This assay was then widely used for the analysis of biological samples and carnitine was found to be distributed, with a few exceptions, throughout nature. Carnitine levels in mammalian tissue were found to vary between 0.1 and a few millimoles per litre [29], with the highest levels recorded in heart and skeletal muscle. In 1957 Fraenkel and Friedman [30] proposed that if a compound was so universally common, and appeared so functionally important to the organism, it should have been identified earlier if it was not synthesized by the organism. Thereafter work began on discovering the endogenous biosynthetic pathway in mammals.

Elucidation of the biosynthetic pathway of carnitine in mammals began in 1961 [31] when studies revealed that the methyl groups of the quaternary ammonium functionality were derived from methionine. The precursors for this part of the carnitine molecule, however, remained unknown with work continuing in this area for the next decade. In 1962 the conversion of γ -butyrobetaine to carnitine was described [32], as was the later discovery of lysine as a precursor of butyrobetaine [33].

In 1973 a biosynthetic pathway of carnitine was elucidated. In animals, protein-bound lysine (stored mainly in muscle tissues) becomes available as peptide residues and is methylated by *S*-adenosylmethionine and a protein methylase before proteolysis liberates ϵ -*N*-trimethyllysine (TML). Oxidation through a further three enzyme-dependent steps converts TML to γ -butyrobetaine aldehyde [34]. Cytosolic hydroxylase then mediates in

the final hydroxylation step to carnitine. This takes place in the liver, brain and human kidney tissue [35], though the liver is the primary site for carnitine synthesis in humans. Tissues lacking the cytosolic hydroxylase enzyme can use the blood circulation to export the γ -butyrobustine precursor to the hydroxylating tissue, but rely on newly synthesized product or dietary intake for their supply of carnitine. Four other micronutrients are required as co-factors by the enzymes involved in the biosynthesis; these are vitamin C, niacin, vitamin B₆ and iron [36]. Deficiencies of these micronutrients as well as of methionine have been shown to reduce carnitine levels in plasma and/or tissue [37].

More recent work, on rat liver mitochondria [38], led to the hypothesis that the inner mitochondrial membrane, being impermeable to CoA and acetyl CoA, required carnitine to transport the acetyl groups in the form of acetylcarnitine across the mitochondrial membrane. Carnitine was also shown to stimulate the oxidation of long-chain fatty acids (palmitate) which led to the theory that carnitine played a role in the transport of other acyl groups [39]. This transport was shown to take place through carnitine translocating activated long-chain fatty acids into the mitochondrial matrix for β -oxidation.

1.2 THE BIOCHEMICAL ROLE OF CARNITINE AND CARNITINE ESTERS.

Carnitine is found in an omnivorous diet but a biosynthetic pathway is also available through methylation by methionine of a lysine-derived carbon chain. The role of carnitine is to act in the transport and metabolism of fatty acids, to maintain a balance between free and esterified CoA, and to remove any excess acyl groups (RCO) from mitochondria. An accumulation of acyl groups is potentially toxic, causing inhibition of enzymes, so carnitine is essential because it is involved in their removal from the mitochondria via acylcarnitines 2, Figure 1.1 opposite page 22 [40].

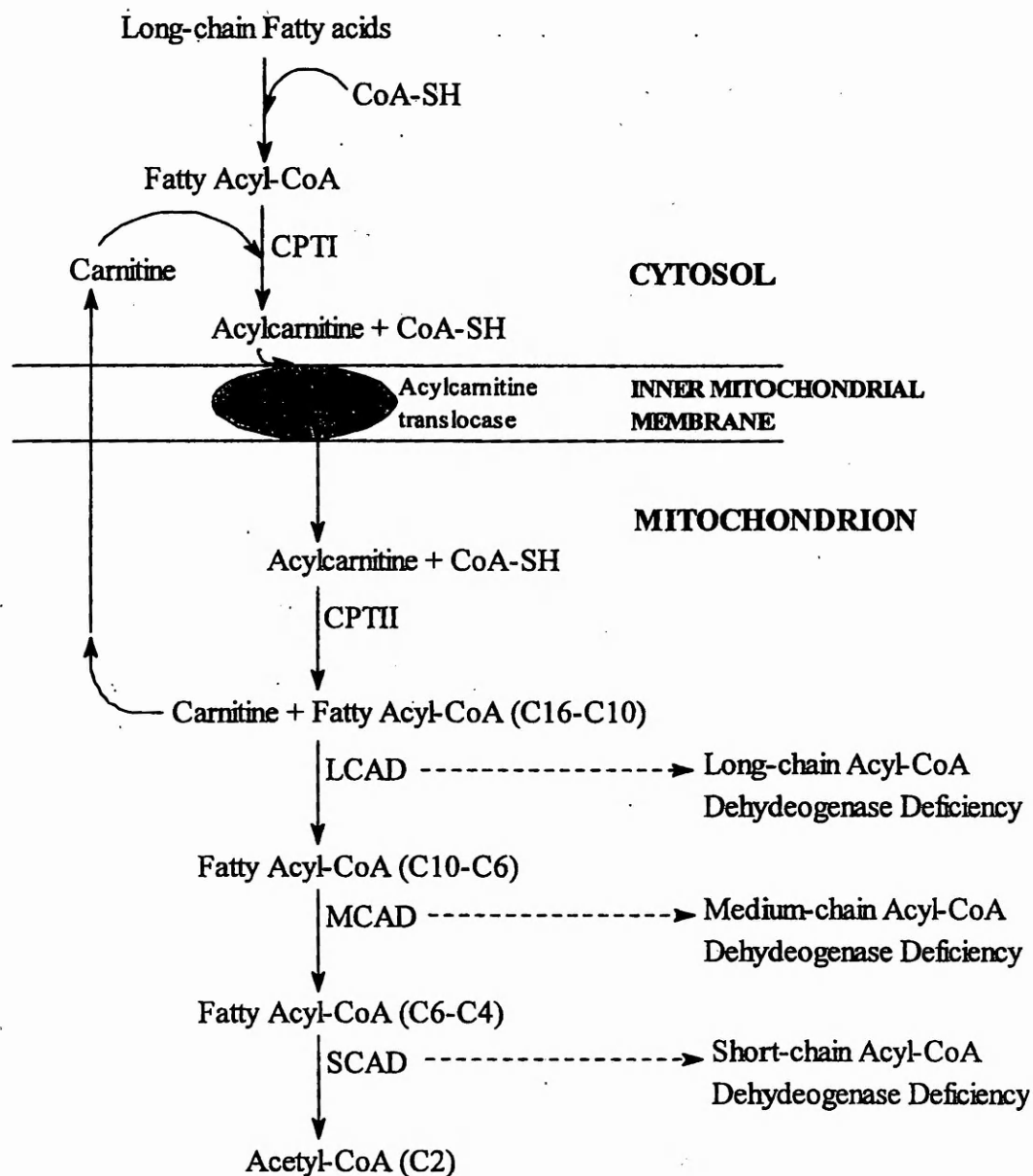


Figure 1.2 Schematic of the activation, membrane crossing and β -oxidation of long-chain fatty acids. Indication also of possible points of problems within the fatty acid oxidation cycle which can give rise to metabolic disorders i.e. MCAD \rightarrow Medium Chain Acyl-CoA Dehydrogenase Deficiency

The process of β -oxidation operates to varying degrees in almost every tissue in the body and acts as the major source of energy production during fasting. The process of β -oxidation is illustrated in Figure 1.2. Long-chain fatty acids are mobilized from the adipose tissue and circulated to the liver and other tissues bound primarily to albumin. This uptake is a concentration-dependent mechanism, which though poorly understood at the moment, may include both saturable carrier-mediated uptake and non-saturable diffusion [41]. These fatty acid substances are then activated to form highly polar fatty acyl-CoA esters in the cytoplasm through the action of the cytoplasmic enzyme acyl-CoA synthetase, in an acylation reaction that is dependent on ATP (adenosine 5'-triphosphate). However, a long-chain fatty acyl-CoA cannot cross the inner mitochondrial membrane directly. Long-chain fatty acyl-CoA esters are carried across the mitochondrial membrane through the sequential action of two carnitine palmitoyl transferase enzymes, CPT I and II, on the outer and inner walls of the inner mitochondrial membrane, and conjugation with carnitine. Medium and short-chain fatty acids can traverse the mitochondrial membrane as free acids and are activated to form acyl-CoA esters within the mitochondrial matrix [42]. Conjugation of long-chain fatty acyl groups with carnitine is brought about by CPT I and the acylcarnitines generated enter the mitochondria where the CPT II enzymes facilitate the regeneration of carnitine and acyl-CoA. Activity of CPT I in the rat was demonstrated as increasing five fold during the initial 24 hours of life, peaking at 2 - 3 days [43] and this activation process has also been observed in the case of humans on a similar time scale. This time scale of activation of the carnitine transport system coincides with the change from the high carbohydrate diet of the foetus to one high in fatty post-partum [44].

The carrier system into and out of the mitochondrion is thought to be identical and the enzymes involved in the carrier system have overlapping chain-length specificities [45]. Carrier enzymes exist for the transport of specific chain-length acylcarnitines, with

equimolar amounts of each transferase being found in the mitochondrial membrane [46]. Acylcarnitine translocatase, a transmembrane protein, is responsible for the passage of the carnitine *O*-acyl esters through the inner mitochondrial membrane [45, 47].

Under normal circumstances, once inside the cell transesterification occurs yielding the starting compounds - carnitine and acyl-CoA. The latter is dismembered, two carbons at a time, by undergoing β -oxidation, eventually producing acetyl-CoA [48, 49]. The breakdown of the fatty acyl chains is governed by a number of enzymes and co-factors and takes place through the closely coupled enzyme system of β -oxidation. Each spiral of the pathway of β -oxidation requires the action of three enzyme types. The first are the group of acyl-CoA dehydrogenase enzymes. These enzymes [50, 51] are chain-length specific acting in long-, medium- and short-chain forms. The role of the acyl-CoA dehydrogenase enzymes is in the insertion of a double bond between the α and the β carbons of the acyl-CoA moiety forming an enoyl-CoA. They also transport the electrons from dehydrogenation to the electron transfer flavoproteins (ETF) [52]. Disorders, which manifest themselves through inhibition of these dehydrogenases, cause a build-up of a specific chain-length acyl-CoA that can have toxic effects. Carnitine acts through conjugation with these acyl moieties to form acylcarnitines which can be identified in biological fluids at abnormally high levels and the chain length of the acylcarnitine will be indicative of a particular enzymic disorder. The second enzyme step is the action of enoyl-CoA hydratase (crotonase) on enoyl-CoA producing L-3-hydroxy-CoA and this is followed by the catalysis of the L-3-hydroxy-CoA to a keto group in an NAD^+ dependent reaction mediated by 3-hydroxy-CoA dehydrogenase [42]. The final cleavage of the α,β bond in the presence of reduced CoA is catalysed by 3-ketoacyl-CoA thiolase. Evidence for chain-length specificity has been proposed for enoyl-CoA hydratase and 3-hydroxy-CoA dehydrogenase in mammalian tissues [53, 54, 55, 56] though this specificity has not

yet been characterised in humans. Acetyl-CoA produced from the spiral of β -oxidation is then used within the tricarboxylic acid cycle (TCA) to produce energy in the form of ATP and also CO_2 and H_2O . To a smaller extent, the kidneys also utilize acetyl-CoA in the formation of ketone bodies, β -hydroxybutyrate and acetoacetate via the hydroxymethylglutaryl-CoA pathway (HMG-CoA). These products are then exported for final oxidation to other tissues e.g. the brain.

Endogenous synthesis of carnitine is not possible for foetuses and neonates due to low activity of butyrobetaine hydroxylase [57, 58]. Even at the age of three months this enzyme's activity, in the liver, has been recorded as 12% of that of adults [58]. Adult carnitines levels are achieved by seven months of age due to increased synthesis and dietary intake [59]. Initially therefore infants must be supplied with the carnitine that they require. Sources of this carnitine include a dependence on the maternal carnitine status in the foetus [57, 59], with placental transfer of the carnitine [60]. This maternal dependence continues in the case of breast fed infants but can be added to or replaced with formula or other dietary supplementation [44].

Other factors which can affect the activation of the carnitine transport system are the presence of certain hormones or the mitochondrial membrane microenvironment [44]. There are characteristic hormone profiles present at birth and these are thought to be specific for the activation of vital metabolic systems post-partum. These include catecholamine release, insulin, glucagon, thyroxine and growth and sex hormones. They are thought to modify protein subunits thus affecting carnitine acyl-transferase and translocate activity. Hormones are also thought to indirectly affect membrane fluidity [44]. Factors such as membrane fluidity, permeability and electro-chemical charge gradients require careful maintenance for the optimal activity of membrane associated proteins [61] such as those involved in the carnitine transport system.

The presence of acylcarnitines in the blood and urine of neonates can characterise a number of inherited metabolic diseases. In patients with metabolic disorders the amount of acyl-CoA being transported is greatly increased and the elevation in acylcarnitines is significant. Di Mauro et al in 1976 [62] reported the first of these disorders. If change in the profile of acylcarnitines can be recognised it may then be possible to identify at which stage of the β -oxidation pathway the breakdown has occurred and thus the disease involved. Most disorders are characterised by recurrent episodes of fasting, coma and hypoglycaemia and in some cases chronic muscle wasting and cardiomyopathy. At the time of acute illness the risk of mortality is high but once the diagnosis is made the prognosis for the patient is excellent. In up to 25% of cases of medium-chain acyl-CoA dehydrogenase deficiency (MCADD) the first episode will prove fatal [42] though some patients may remain undiagnosed until another member of the family is affected and further tests carried out. In the defects, which involve β -oxidation, a secondary carnitine deficiency is presented as a reduction in the total carnitine level in plasma and there is an increase in the fraction of esterified carnitine [63].

A number of reviews on the analysis of biological samples for acylcarnitines have been published [64 - 69].

1.3 ANALYSIS OF FREE AND TOTAL CARNITINE.

Carnitine is frequently measured in biochemical, clinical or nutritional studies.

Estimation of total acylcarnitines usually involves hydrolysis of all acylcarnitines to carnitine followed detection and measurement by a number of methods. There are a number of review articles covering this area of study [70 - 73].

In humans, plasma concentrations of carnitine are relatively stable at $46 \pm 10 \mu\text{mol/L}$ of which about 15% are in the form of acylcarnitines, mostly acetylcarnitine [45]. Reports of normal concentrations of total carnitine and free carnitine in the serum of healthy men were 62.2 ± 4.4 and $55.9 \pm 4.0 \mu\text{mol/L}$, respectively, and that of healthy women was 55.9 ± 6.3 and $46.6 \pm 7.1 \mu\text{mol/L}$ respectively [74]. Whole blood is recorded as containing 50% more carnitine than occurs in plasma [75]. The levels of free carnitine in plasma are thought to reflect the carnitine tissue levels and are considered abnormally low if they fall to less than $20 \mu\text{mol/L}$. Studies have indicated that although carnitine is found in all particulate blood components, higher concentrations are detected in white blood cells. Red blood cells and plasma have the same concentrations of free carnitine though in the former short-chain acylcarnitine is enriched with its ratio to free carnitine up to 1.0 [76]. Deficiency of carnitine can cause a number of clinical symptoms including myopathy, hypotonia and hypoglycaemia [77].

Enzymes techniques have perhaps been most widely utilized in the detection of carnitine and acylcarnitines in biological fluids, tissues, pharmaceuticals and a variety of foods. A widely used enzyme for assaying these compounds is carnitine acetyl transferase (CAT). Reactions coupled with the CAT enzyme are common and there are a number of means of detection including UV, radiolabelled detection or a combination with high-performance liquid chromatography (HPLC). The latter provides enantiomeric and structural specificity and, when coupled with radiolabelling, highly sensitive assays [78].

Carnitine can be assayed in a number of ways. One of the more common is radioenzymic exchange assay (REA) which uses radiolabelled acetyl-CoA ($1\text{-}^{14}\text{C}$ -acetyl-CoA) as the reaction substrate for the CAT enzyme and measures the $1\text{-}^{14}\text{C}$ -acetylcarnitine produced [79, 80]. This method has been used and modified to assay the carnitine concentration in

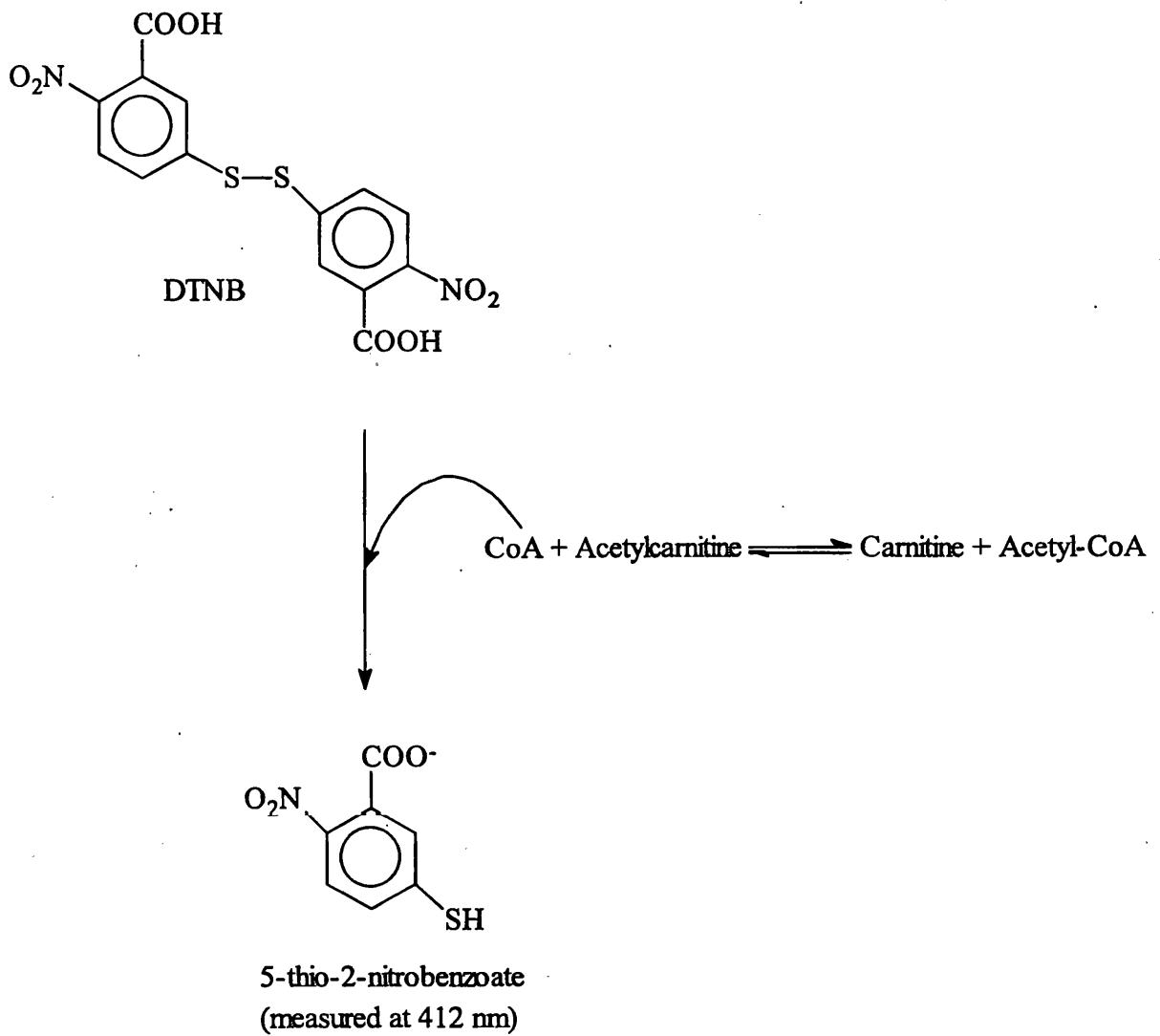


Figure 1.3 Enzymic conversion of carnitine and acetyl-CoA into acetylcarnitine and CoA, and the further reaction of CoA with DTNB, to produce the 5-thio--nitrobenzoate anion for radioenzymic analysis.

rat bile [81], urine, plasma and/or tissue and human skeletal muscle from needle biopsies [82]. Radioenzymic methods have been used for the measurement of carnitine, short-chain acylcarnitines and long-chain acylcarnitines in plasma and tissue with between- and within-batch precision of 10.4 and 7%, respectively [83].

A method has also been used based on the reaction of the CoA liberated with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) [84, 85, 86]; a scheme of this method is presented in Figure 1.3. Hydrolysis of acylcarnitines to free carnitine is the initial step in this process, as described, then in the presence of acetyl-CoA and carnitine acetyltransferase; the carnitine is converted to acetylcarnitine, producing CoA. The CoA reduces added DTNB to the yellow 5-thio-2-nitrobenzoate anion in proportion to the amount of L-carnitine. The ion is measured at 412 nm [74]. The method has been applied to the analysis of the carnitine content of serum, cerebrospinal and seminal fluids [87] and human tissues. Plasma carnitine levels have also been determined by column chromatography [88] combined with DTNB detection [89].

Conversion of carnitine to resorufin, involving the use of immobilized dehydrogenase and diaphorase enzymes, has been used with fluorometric monitoring [90]. The coupling of the CAT reaction to *N*-[p-(2-benzimidazolyl) phenyl] maleimide (BIPM) allows the detection of the fluorescent CoA-BIPM which is present proportionately to carnitine [91, 92]. Tissue labelled with [1-¹⁴C]palmitate has been treated with a phospholipase allowing the 2-dimensional TLC (acidic then basic solvent) detection of ¹⁴C-labelled long-chain acylcarnitines. These are then hydrolysed freeing carnitine for enzymic assay [93, 94].

Enzymic methods have also been applied to the assay of carnitine and acylcarnitines in foodstuffs including milk and milk products [95]. Raw, pasteurised and skimmed milk,

yoghurt, butter and cheese are among the dairy products analysed in this manner [95]. A reversed-phase HPLC technique to separate carnitine and acylcarnitines from a biological matrix utilises a step gradient to provide baseline resolution of acylcarnitines (individually or by class) and allows quantification by using a sensitive radioenzymic assay [96].

The enzymic techniques described above have also been used in conjunction with centrifugal analysis. This technique provides a spectrophotometric assay of free and total carnitine in plasma ultrafiltrates and may be suitable for routine application in many hospital laboratories [74, 97, and 98]. Analysis has been carried out on serum L-carnitine, with recovery of carnitine from spiked serum reported as 93% [97]. Analysis using DTNB has also been applied to measure free and total carnitine in human tissue [99]. The CAT enzyme reaction above has also been followed by reaction of CoA with 2-oxoglutarate to produce succinoyl-CoA. Catalysed by 2-oxoglutarate dehydrogenase, this product reduces nicotinamide adenine dinucleotide (NAD), which is then monitored spectrophotometrically [100].

Both gas chromatography (GC) and liquid chromatography (particularly HPLC) have been used for the determination of carnitine in a variety of matrices. For HPLC, the chromophoric properties must be enhanced for successful analysis. For analysis by HPLC once the compounds of interest are extracted and dried conversion to 4-bromophenacyl ester derivatives is a primary derivatization choice [101]. The same derivative has also been used with reversed-phase HPLC for urine analysis [102]. Alternatively, derivatization has also been achieved through formation of a fluorescent ester, through a pre-column reaction with 9-anthryldiazomethane [103] followed by HPLC. Stoichiometric conversion to CoA by carnitine acetyltransferase and analysis of

the formed CoA by HPLC on C₈ reversed-phase columns [104, 105] has also been reported.

For GC, derivatization prior to separation is required to volatilize the molecules. The involatility of carnitine for GC analysis can be overcome by conversion to 4-butyrolactone by reduction with sodium borohydride under basic conditions [106, 107, 108]. Packed-column GC and flame ionization detection (FID) were used to evaluate carnitine levels in mature rat epididymis samples [108] and milk [107]. Following solvent extraction, work-up and derivatization, GC was also used to determine carnitine in rabbit tissue [106].

Mass spectrometry, and its linking with techniques such as those described above, opened the area for more specific determination of detected acylcarnitines. Pyrolysis is an important process in mass spectrometric analysis of acylcarnitines [109]. Carnitine and acylcarnitines undergo two major competing pyrolytic reactions under electron ionization conditions. Elimination of water from carnitine, or of the carboxylic acid from an acylcarnitine, precedes intramolecular displacement of trimethylamine and formation of 2(5*H*)-furanone. Secondly, the same intramolecular displacement can occur with formation of an acyloxy-substituted γ -lactone and trimethylamine [109].

In the case of isotope dilution assay analyte which has been labelled with a stable isotope is used as an internal standard. This technique serves to reducing variation found within an assay. Different known amounts of analyte are diluted with a constant amount of the internal standard to generate a calibration curve against which samples containing unknown quantities of analyte and fixed internal standard can be back-calculated. This technique coupled with fast atom bombardment (FAB) ionization in the positive-ion mode with tandem mass spectrometry (MS/MS) has been used to evaluate total and free-

carnitine levels in urine and plasma [110]. The behaviour of carnitine in the solid and solution phases has been investigated through bombardment with atoms or ions followed by negative-ion or positive-ion mass spectrometry [111, 112]. Gas-phase chemistry of carnitine was also studied by MS/MS.

Carnitine in human and rat fluids has been measured using a carnitine-specific mutant of the enteric yeast *Torulopsis bovina* which has a response threshold to carnitine of 100 pg/ml. A turbidimeter is used to measure growth, as absorbance units, and the method can be used to measure acid-soluble and total (acid- and alkali-soluble) carnitine [113]. Lipid-bound carnitine was assayed after precipitation with $\text{Cl}_3\text{CCO}_2\text{H}$. The average recovery of free carnitine was 95% and that of lipid-bound carnitine was in the range of 76 - 95% [114].

1.4 METHODS OF ANALYSIS FOR ACYLCARNITINES.

A wide variety of techniques have been utilised in the analysis of carnitine esters. These include enzymic methods similar to those described above for the characterization of free and total acylcarnitines. A number of separation techniques have been used for these analyses including thin-layer chromatography (TLC), HPLC, GC and other separation techniques, operated independently or coupled with single or multiple mass spectrometric detectors.

In the experimental work discussed in this thesis the main technique addressed is the analysis of acylcarnitines by gas chromatography either operated independently or coupled with a mass spectrometer. The involatile nature of the zwitterionic carnitine and acylcarnitines makes their GC analysis impossible without derivatization, thus making it necessary for their conversion to more volatile compounds. Conditions necessary to

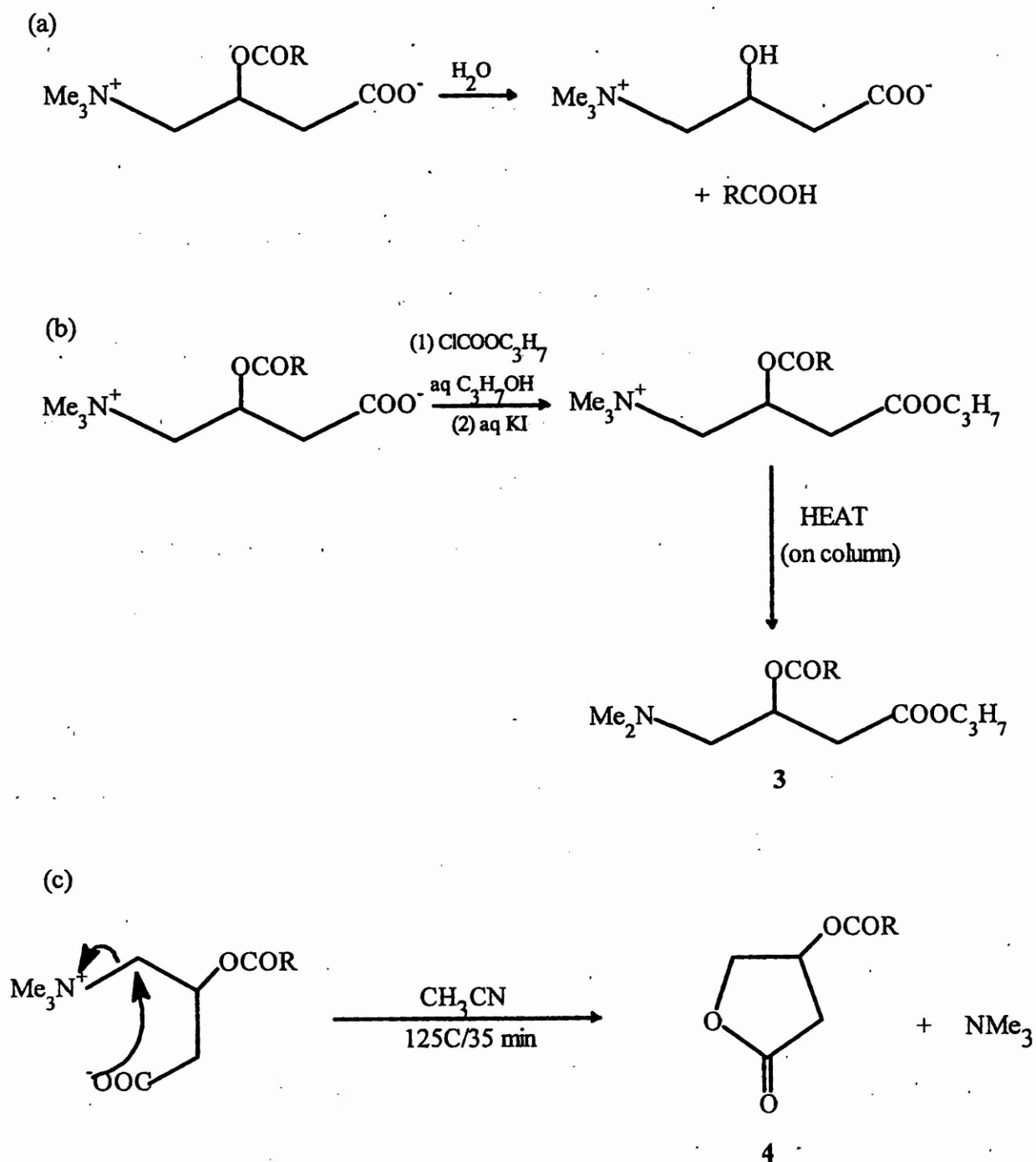


Figure 1.4 Different schemes for converting acylcarnitines into volatile derivatives for GC and/or GC/MS analysis: (a) hydrolysis to carboxylic acids, (b) a dequaternization (*N*-demethylation) route 3, and (c) cyclization to lactones 4.

increase the volatility of acylcarnitines have been investigated by a number of groups. It has been reported that carnitine can be converted into a γ -butyrolactone prior to GC analysis [106, 107, 108]. This intramolecular displacement of trimethylamine and formation of an acyloxy-substituted γ -lactone occurs when carnitine and the higher acylcarnitines are subjected to electron ionization mass spectrometry [109]. Cyclization through heating is the method, which has been advanced in this thesis for gas chromatographic examination of a wide range of acylcarnitines [115].

The first procedure illustrated in Figure 1.4 is the hydrolysis of carnitine esters to yield carboxylic acids, which are then subjected to GC analysis [67, 116]. Identification of nanomolar amounts of short-chain acyl residues in this way has several drawbacks. With the necessary extensive work-up, it is time-consuming. There may also be some ambiguity in the result because the memory of the carnitine origin of the acyl groups is not retained once hydrolysis has taken place. In this way contaminating carboxylic acids would give misleading data; hence the need for exhaustive isolation procedures.

Modifications to this method are required before it can be applied to longer chain acylcarnitines [117]. Even so, the method has been applied successfully to the quantification of water-soluble acylcarnitines in rat tissue [118] and to the identification of aliphatic short-chain acylcarnitines in beef heart [119]. In mammalian tissues, acetyl-, propanoyl-, 2-methylpropanoyl-, butanoyl-, 2-methylbutanoyl-, 3-methylbutanoyl- (isovaleryl-), 2-methylbut-2-enoyl- (tiglyl-) and hexanoyl- carnitines were found. Other acylcarnitines were also identified or tentatively identified in these studies.

More effective approaches are illustrated in Figure 1.4 (b) - (c), shown opposite. In both of these derivatization schemes, the end product retains a memory of its origin inasmuch as a diagnostic portion of the carnitine structure occurs in the derivative. Hence, the carnitine origin of the acyl residue is unambiguous in the subsequent analysis. The

application of the *N*-demethylation [120] and lactonization approaches [115, 121] are discussed later.

A problem for any gas chromatographic method of analysing for acylcarnitines is the lack of structural information in the resulting chromatogram. Complex chromatograms produced by analyses of this nature mean that in many cases there cannot be total confidence in structural assignments based solely on retention times. Coupling of the gas chromatograph with a mass spectrometer can overcome this shortfall, if necessary. Despite the potential difficulties of gas chromatography alone, an assay for urinary medium-chain acylcarnitines has been developed which produces readily interpretable and uncomplicated chromatograms that are said to circumvent the requirement for mass spectrometry [122]. In this study, GC was used directly for assay of urinary medium-chain fatty acylcarnitines; that is, the method does not require a separate derivatization step. Rather, the acylcarnitines are allowed to decompose thermally in the hot injection zone of the GC system. At 280 °C, each acylcarnitine appears to undergo an ester pyrolysis reaction, giving the carboxylic acid corresponding to each acyl group. It is these acids that elute through a GC column coated with the polar stationary phase, PEGA. Given that fatty acids are the actual substances detected, it is important that free acids do not contaminate the urine extracts containing acylcarnitines. In the method described [122], carboxylic acids are extracted from the urine with chloroform prior to extraction of acylcarnitines into butan-1-ol. However, it is reported that only about 60% of the acids are so removed and it has also been proposed that some of the acylcarnitines will dissolve in chloroform [123] thus giving rise to poor sensitivity. Despite the inherent weaknesses, the method enabled detection of octanoylcarnitine in a symptomatic individual with medium-chain CoA dehydrogenase deficiency and in two asymptomatic siblings following administration of carnitine.

The coupled technique (GC/MS) usually provides lower limits of detection and greater structural specificity compared with GC alone. GC/MS can now be carried out routinely on any one of a number of inexpensive and simple-to-use benchtop systems, thus opening the technique for biochemical and neonatal screening laboratories to obtain acylcarnitine profiles in biological matrices.

Acylcarnitines can be derivatised in a number of ways to render them suitable for analysis by GC/MS. One involves direct esterification using propyl chloroformate in aqueous propan-1-ol in the presence of pyridine and requires only 5 minutes at room temperature. After addition of potassium iodide, the resulting acylcarnitine propyl ester iodides are extracted into chloroform and their subsequent *N*-demethylation can be brought about conveniently in the hot injector port of the GC/MS system (260°C), causing the formation of volatile derivatives **3** shown in Figure 1.4 (b), opposite 34. These *N*-demethylated acylcarnitine propyl esters are well separated on a gas chromatographic stationary phase of DB-1 and are readily detected and identified by their methane chemical ionization mass spectra which are characterized by abundant $[M + H]^+$ ions and several diagnostic fragment ions. The detection limits of medium-chain acylcarnitine standards ($C_4 - C_{12}$ side-chains) were demonstrated to be below 1 ng of starting material when using selected ion monitoring of $[M + H]^+$ ions and a common fragment ion. By this method, seven acylcarnitines (with $C_{5:0}$ to $C_{10:1}$ side-chains) have been characterized in the urine of a patient suffering from medium-chain acyl-CoA dehydrogenase deficiency [120]. The same GC/MS method also revealed that octanoylcarnitine, not valproylcarnitine, was the most abundant medium-chain carnitine ester excreted by a patient treated with valproic acid [125]. This latter result is pertinent to findings in this thesis (Section 3.3).

Alternatively, acylcarnitines can be extracted from urine either by ion-exchange chromatography [115, 121] or by solvent extraction [125] and heated in acetonitrile for about 30 min at 125°C in the presence of *N,N*-diisopropylethylamine to effect cyclization to a lactone 4 (Figure 1.4 (c) , opposite page 34). Such acyloxylactones elute readily on a DB5 GC column and can be identified by their chemical ionization and/or electron ionization mass spectra. Using this method it was found that monocarboxylic acylcarnitines from acetylcarnitine (C₂ acyl chain) to octadecanoylcarnitine (C₁₈ acyl chain) can be isolated from urine with recoveries of over 80%. To obtain such recoveries, different methods of extraction had to be used for different ranges of acylcarnitines. For shorter chain acylcarnitines (C₂ to C₈ side-chains) an ion-exchange procedure was recommended. Acylcarnitines with acyl chain length C₈ to C₁₂ were reported to be isolated most effectively from urine by solvent extraction with butan-1-ol as long as the urine had been acidified to about pH 2. For long-chain acylcarnitines (C₁₀ to C₁₈ acyl chains) solvent extraction of unacidified urine with hexan-2-ol was particularly simple and effective [125]. These results may have implications for any method of acylcarnitine analysis that requires prior purification.

The lactonization and GC/MS approach has been applied to several disorders of organic acid metabolism associated with abnormalities in the levels of urinary acylcarnitines, such as medium-chain acyl-CoA dehydrogenase deficiency [115, 121], propionic acidemia, isovaleric acidemia, multiple acyl-CoA dehydrogenation deficiency [121] and long-chain acyl-CoA dehydrogenase deficiency [125]. It has also been used to detect metabolites of exogenous compounds, as with 3-phenylpropanoylcarnitine in babies who had received a 3-phenylpropanoic acid load, and an ester thought to be 2-propyl-3-oxovalerylcarnitine in the urine of an infant undergoing valproic acid (2-propylvaleric acid) therapy [115, 126,

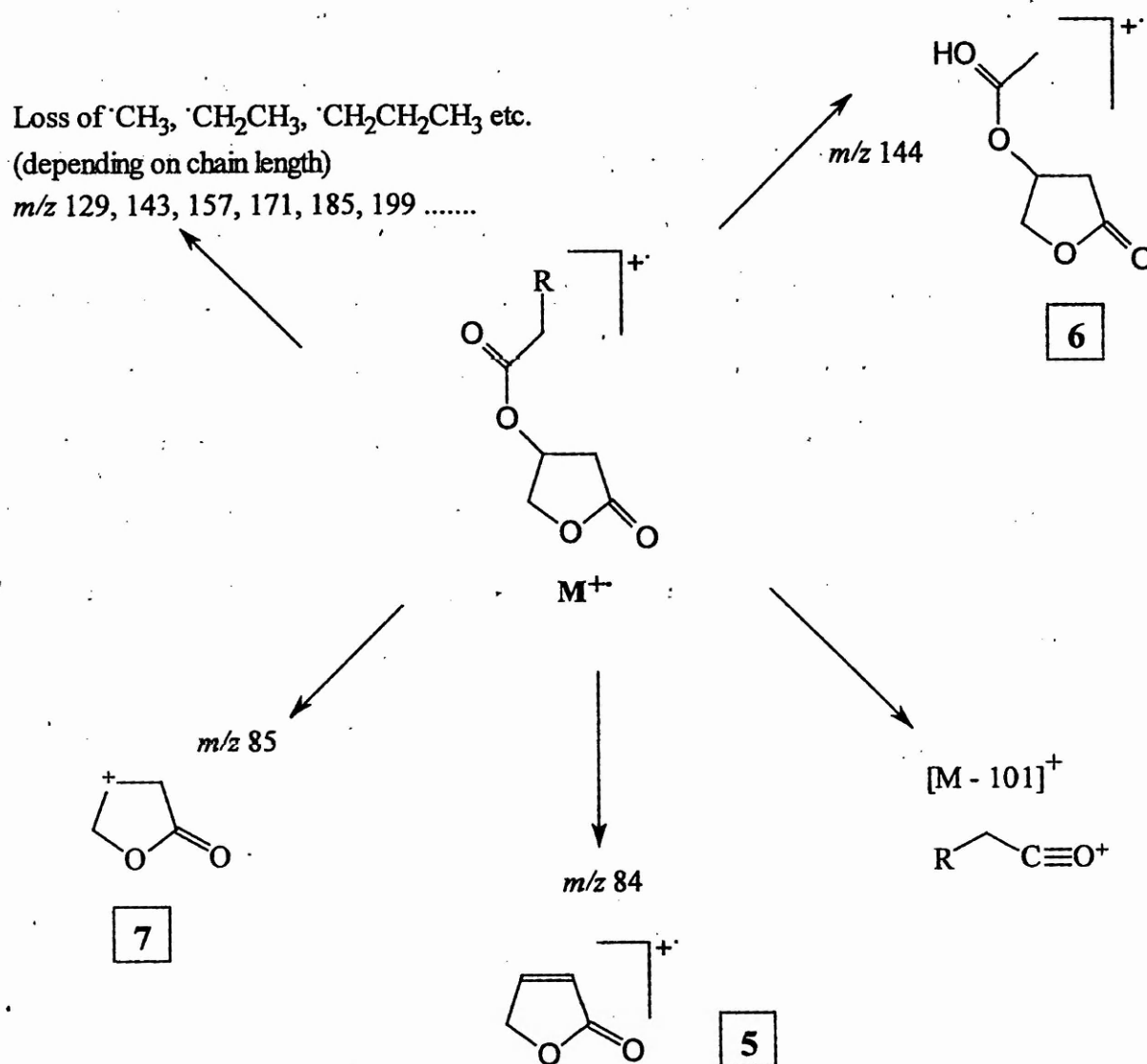


Figure 1.5 Characteristic fragment ions from the EI mass spectra of acyloxylactones. These characteristic fragments are used throughout the work described in this thesis to identify diagnostic acyloxylactones

127]. A more thorough analysis of valproate metabolism is considered later in this thesis (Section 3.3.1)

It has been found in the course of this work that, at the derivatization stage, more prolonged heating or higher temperatures cause some decomposition to fatty acids, and that the use of heated GC injectors at over 230°C facilitates ester pyrolysis as observed by others [122]. Cold on-column injectors or split/splitless injectors at 230°C were preferred [115]. However, another worker has found that, high injection temperatures i.e. in the region of 280°C, a degree of ester pyrolysis occurs, resulting in peaks for carboxylic acids but that the major reaction is lactonization as in Figure 1.4 (c), opposite page 34 [123]. This on-column version of the cyclization approach promises to be a fast and convenient method for GC/MS of acylcarnitines but does not allow much control over the cyclization conditions. In addition, efficiency of cyclization may vary with the conditions and the type of injector. The injection system most widely regarded as providing the best quantitative results (cold on-column injection) is not compatible with on-line cyclization as high temperatures are required.

The electron ionization (EI) mass spectra of the acyloxylactones are not ideal for identification purposes because the molecular ions are of low abundance and often absent, particularly when dealing with trace amounts from biological samples [115, 121]. However, the fragment ions are characteristic of structure (Figure 1.5). Identification of an unknown acylcarnitine derivative was based on its GC retention time and matching of its EI mass spectrum against a library of standard spectra generated from synthesized acyloxylactones.

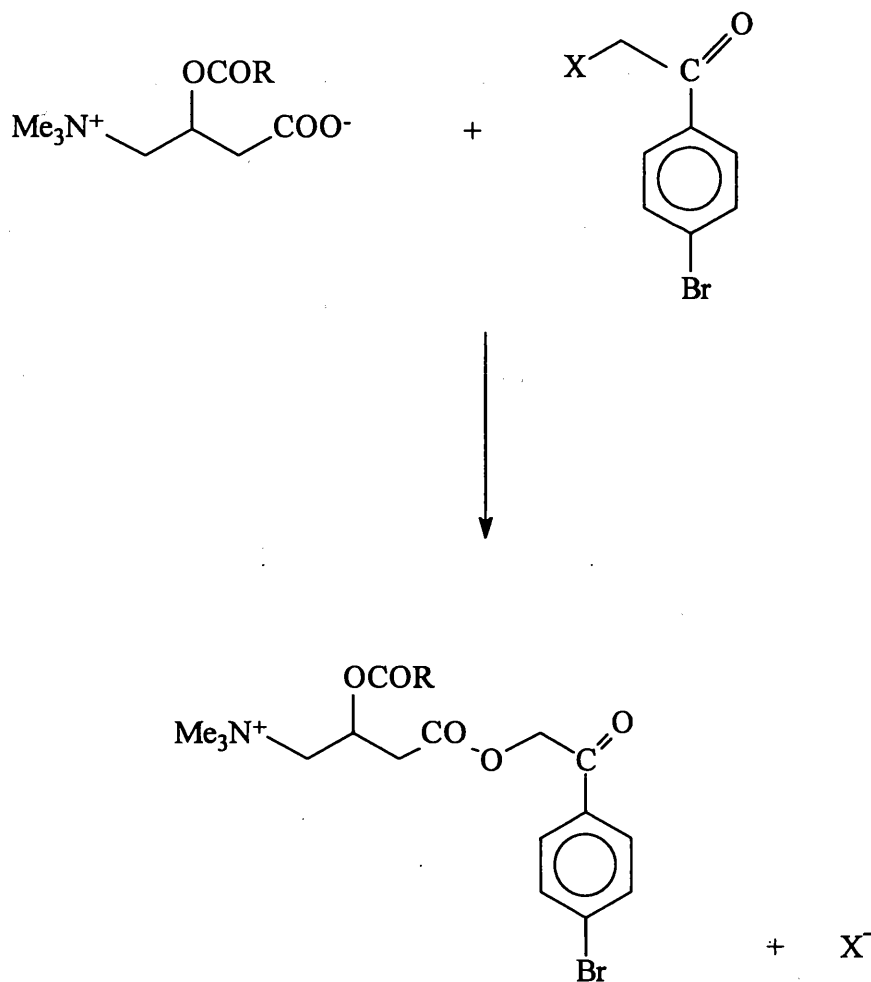


Figure 1.6 Formation of chromophoric 4-bromophenacyl esters of acylcarnitines ($\text{X} = \text{Br}$ or OSO_2CF_3) prior to HPLC analysis on a silica column, in a mixed partition and ion exchange mode.

A high-resolution liquid chromatographic technique is available for acylcarnitines in the shape of HPLC. A key problem for this technique is the limit of detection of acylcarnitines as naturally occurring acylcarnitines are only weakly chromophoric (λ_{max} is about 210 nm) and are neither electrophoric nor fluorophoric to any useful degree. It is therefore necessary to enhance this property to increase the detection of eluting acylcarnitines. Derivatization, radiochemical detection or coupling the system to a mass spectrometric detector have been used to increase sensitivity structural specificity.

A simple and effective method for separating carnitine and acylcarnitines from a biological matrix (rat liver tissue) prior to quantification by radioenzymic assay is reversed-phase step-gradient HPLC. The chromatographic method, with spectrometric detection at 210 nm, also permits resolution of long-chain acylcarnitines in the presence of large excesses of carnitine and short-chain acylcarnitines [96].

The commonest derivatization strategy for the HPLC analysis of carnitine and its esters involves the formation of chromophoric 4-bromophenacyl esters (Figure 1.6). Other methods include derivatization with 4-bromophenacyl trifluoromethanesulphonate (4-bromophenacyl triflate) in acetonitrile containing *N,N*-diisopropylethylamine. This is followed by separation by reversed-phase ion-pair HPLC [128, 129, 130], and the determination of total carnitine in human urine by base hydrolysis, ion-exchange purification of carnitine, derivatization, chromatography on Radial-Pak C₁₈ of 10 μm particle diameter, and spectrophotometric detection at 254 nm [131].

Reaction of acylcarnitines with 4-bromophenacyl triflate in the presence of magnesium oxide as base also produces the same derivatives. The ester derivatives produced have been separated by HPLC on a silica column, in a mixed partition and ion-exchange mode. Using this method, carnitine and acylcarnitines in biological media can be measured in

100 µl samples, with a detection limit below 1 µmol/l [101]. Isolation of urinary carnitine and acylcarnitines using ion-exchange columns followed by derivatization to the 4-bromophenacyl derivatives by reaction with 4-bromophenacyl bromide in the presence of a crown ether and potassium ions under carefully controlled conditions has been reported [127]. The derivatives were then subjected to reversed-phase HPLC (25 x 0.5 cm i.d. packed with 5 µm Spherisorb Octyl) with a mobile phase of 7% 0.25 M, pH 5.8 trimethylamine phosphate buffer in a water: acetonitrile gradient. Detection was by UV absorption at 254 nm and urinary propanoylcarnitine was observed, as expected, in a neonate with propionic academia. Similar methodology has been employed to study the effects of long-chain fat loads on two asymptomatic patients with medium-chain acyl-CoA dehydrogenase deficiency with octanoylcarnitine, hexanoylcarnitine and acetylcarnitine detected at 260 nm [133].

Carboxylic acid analysers, comprising reversed-phase HPLC with post-column derivatization, have been investigated for studying inherited metabolic disorders by measuring urinary acylcarnitines [134, 135, 136]. The derivatization carried out on-line involves the reaction between the carboxylic acid group in the acylcarnitine and 2-nitrophenylhydrazine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. This is then followed by colorimetric detection. Anion-exchange chromatography is required to remove interfering carboxylic acids from urine before the HPLC analysis can proceed. Linear calibration curves over the range 30 - 1000 nmol/ml were obtained for carnitine, acetylcarnitine, glutaryl carnitine and propanoylcarnitine, as appropriate for urinary levels [135, 136].

A method for detecting and quantifying acylcarnitines by HPLC using a strategy based on radioisotopic exchange has been widely described [67, 125, 137, 138, 139]. Briefly, high specific activity L-[³H]- or L-[¹⁴C]carnitine is incorporated into the acylcarnitine pool in

a sample by enzymic exchange. The acylcarnitines must be substrates for the carnitine acyltransferase(s) used and the enzyme(s) must be totally free of acyl-CoA and acylcarnitine hydrolytic activity. Under these conditions picomolar levels of individual acylcarnitines were detected after isotopic equilibrium is established by subjecting the radioactive acylcarnitines to either HPLC or thin-layer chromatography. After separation, the amounts of radioactivity in the acylcarnitines are measured and the quantity of individual acylcarnitines can be calculated, for example, from the specific activity of the initial total carnitine pool.

The sensitivity and specificity of the radioisotopic exchange/HPLC method for detecting urinary medium-chain acylcarnitines has been found to be sufficient for the diagnosis of medium-chain acyl-CoA deficiency. Over one hundred urine specimens from 75 controls and children with metabolic diseases (in the asymptomatic state without carnitine loading) were analysed in a blind experiment. All 47 patients with MCADD were correctly diagnosed using the criterion that the peak areas of octanoylcarnitine or hexanoylcarnitine are larger than those of other medium-chain acylcarnitines. However, patients receiving valproic acid or a diet enriched in medium-chain triglycerides can also test positive for MCADD by this criterion, so successful application of the method requires a knowledge of medium-chain triglyceride or valproic acid administration [125].

On-line radiochemical detection for reversed-phase HPLC also provides a means of detecting acylcarnitines that are metabolic products of radiolabelled precursors in tracer studies. For example, oxidation of [^{14}C]hexadecanoate by normal human fibroblast mitochondria [140] and by rat skeletal muscle mitochondria [141], and metabolism of [^{14}C]ketoisoleucine by rat liver mitochondria [142] have been studied in this way. In normal human fibroblast mitochondria, only saturated acylcarnitine esters were detected, supporting the concept that the acyl-CoA dehydrogenase step is rate-limiting in

mitochondrial β -oxidation. Incubation of fibroblast mitochondria from patients with defects of β -oxidation show different profiles of intermediates, while mitochondria from patients with defects in electron transfer flavoprotein and electron transfer flavoprotein: ubiquinone oxido-reductase are associated with slow flux through β -oxidation and accumulations of long-chain acyl-CoA esters and acylcarnitines. As expected, elevated levels of saturated medium-chain acylcarnitines were found in the incubations of mitochondria from medium-chain acyl-CoA dehydrogenase deficient patients. The authors rightly state that radio-HPLC of intermediates of mitochondrial fatty acid oxidation is an important new technique to study the control, organisation and defects of the enzymes of β -oxidation [140].

In an entirely different approach, urinary carnitine esters can be converted enzymically into CoA esters with carnitine acetyltransferase. This is followed by separation of the resulting CoA esters on a radially compressed cartridge of Radial-Pak C₈ with a mobile phase containing 0.025 M tetraethylammonium phosphate in a linear gradient of 1% - 50% methanol [143]. Spectrophotometric detection at 254 nm was utilized for quantitative investigations of propionic, methylmalonic and isovaleric acidemias. The enzymic conversion approaches quantitative yields for acetyl and propanoyl esters as long as large amounts of carnitine are not present. This potential problem is not usually serious because acidemia patients produce little free carnitine in their urine [143]. A mixed chromatographic matrix of calcium phosphate supported on macroporous silica microparticles, which has similar selectivity and chemical inertness to hydroxyapatite and mechanical resistance to the pressures generally used in HPLC, has been used to separate biomolecules such as carnitine derivatives and sugars [144]. Finally in this discussion of HPLC, a new method for the determination of acetyl-D-carnitine in the L-enantiomer by enzymic reaction has been reported [145]. The D-isomer was converted stereoselectively

by electric eel acetylcholinesterase into D-carnitine and then separated and determined by ion-pair reversed-phase HPLC.

The techniques covered in this section share with gas chromatography two key disadvantages. The chromatographic process is inherently sluggish and hence does not lend itself to population screening, and neither GC nor HPLC provides structural information on the eluting analytes. The latter deficiency can be addressed, as with gas chromatography, by coupling with mass spectrometry. In addition, the application of LC/MS obviates the need for derivatization with a chromophoric group.

Perhaps the most well developed method to date, which addresses some of the problems of alternative methods, is one consisting of a combination of FAB with either single or tandem mass spectrometers [146]. FABMS is a method whereby a beam of atoms or ions of high translational energy is directed at the surface of a solution of the sample in a suitable liquid matrix [65]. Glycerol has been used as the liquid matrix, for experiments described in this thesis, to provide sensitivity and stability to the FAB ionisation stage. In the case of free carnitine and acylcarnitines the presence of the ammonium functional group accounts for the sensitivity of FABMS [147]. Recently, electrospray coupled with single or tandem MS has superseded FAB in this approach and this methodology will be discussed in detail in Chapter 6.

Tandem mass spectrometry (MSMS) when coupled with FAB and esterification of the carboxylic acid functionality of acylcarnitines [148, 149] increases both selectivity and sensitivity. FABMSMS monitors a controlled fragmentation giving rise to a fragment at m/z 99 from acylcarnitine methyl esters ($C_2 - C_{18:1}$) and looks to a mass range (m/z 200 - 500) for compounds which have given rise to this particular fragmentation, this is termed a precursor ion scan. Using this technique it is reported that fifteen specific disorders of

branched-chain amino acid catabolism can be diagnosed via acylcarnitine analysis [65]. Although this method has many advantages in terms of samples throughput, analysis of urine, blood spots and plasma and its simplicity, isolation of isomers which depends on partition rather than just mass differentiation requires further confirmational analysis using other techniques and as with other methods the need for derivatization is not avoided. The instrumentation required to carry out the type of analyses discussed here is much more complex than a bench-top system and due to its high initial financial outlay is not widespread in hospitals.

Thin-layer chromatography has been used in two significantly different ways. The first is as a fractionating technique prior to application of an analytical measurement [150, 151] and the second as a method of directly analysing carnitine and its esters [152]. Picomole limits of detection have been reported for short-chain acylcarnitines with quantification by radioisotopic-exchange using high-performance liquid or thin-layer chromatography [132]. Two-dimensional TLC has also been applied to the analysis of acylcarnitines [153] as has a planar-layer version of HPLC known as overpressured-layer chromatography. The latter technique combines advantages of high-performance TLC and HPLC [154, 155, 156] with carnitine levels determined to 3 μ g. Evaluation of short-chain acylcarnitines (C₂ - C₅ acyl groups) using combinations of paper chromatography (butan-1-ol:acetic acid: water - 8:1:1) followed by hydrolysis and enzymic assay have allowed detection of this group of acylcarnitines at nanomolar levels [157]. Although analysis using TLC coupled to a mass spectrometer has been reported [158], applications of this technique are not widespread. While allowing the detection of acylcarnitines such methods are cumbersome and provide only limited structural information and resolution when compared to other available methods.

Nuclear magnetic resonance spectrometry has also been used for the analysis of acylcarnitines. When using the technique of NMR spectroscopy for the analysis of acylcarnitines a high-field instrument must be used to override inherently poor sensitivity. In this way NMR can be a fast, powerful technique for monitoring metabolic disorders in neonates, [159, 160] with millimolar level accumulation of substances monitored by ^1H NMR spectroscopy. One problem posed by this method is that while it can be applied to detection of major urinary acylcarnitines, trace levels of these molecules in a more complex matrix would not be detected.

^1H NMR spectroscopy using a new Karplus equation for the observed vicinal coupling constants has provided an insight into the conformations of acylcarnitines in an aqueous matrix during interaction with the CAT enzyme. The relative energies of conformers suggest that carnitine and acetylcarnitine adopt a folded form for binding to the enzyme [161]. ^1H NMR spectroscopy has also been used to measure the enantiomeric excess of carnitine both directly [162] and indirectly [163].

1.5 AIM OF THIS PROJECT

This thesis will outline the development of methodology towards the analysis of acylcarnitines from a number of biological matrices. Its aim is to improve the detection of acylcarnitines from biological fluids making the analysis suitable for use on instrumentation available in the hospital laboratory. The application of a number of analytical and mass spectrometric techniques, including GC, GC/MS and electrospray mass spectrometry (ESI/MS), in the analysis of acylcarnitines, gives a snapshot of metabolism at the time the sample is acquired and a hope that their future use could aid in the diagnosis of SUDS and the detection of neonatal disorders of fatty acid oxidation.

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CHAPTER 2

A BRIEF SURVEY OF THE MAIN ANALYTICAL TECHNIQUES USED IN THIS PROJECT

2.1 GAS CHROMATOGRAPHY

Gas chromatography was the main analytical technique used in the project for separating mixtures of acylcarnitines at trace level from biological matrices. In the case of GC, the sample is transported in a gaseous phase through a SGE BP5 fused silica capillary column. The columns used were 12 - 25m long with 0.2 - 0.3 mm internal diameter (ID). Separation depends on the differing partition coefficients of the components of the sample (i.e. the distribution of analytes between the gas and stationary phase). The carrier gas in the work described was helium and was used at a constant flow rate.

A Carlo Erba 5300 Mega series gas chromatograph with flame ionization detection was used with an SGE BP5 fused-silica capillary column, 24.5 m x 0.33 mm I.D. and 0.5 μ m film thickness. Helium, 35 cm/s linear velocity, was used as the carrier gas. The hydrogen and air inlet pressures were 70 and 120 kPa respectively. The temperature of the detector was maintained at 280 °C. For each analysis, the temperature program for the oven was 87 °C (on injection) to 250 °C at a rate of 10 °C/min. On reaching 250 °C the oven was maintained at this temperature for 15 min. Cold on-column injections (Grob type injector) were performed with a 5 μ l Hamilton syringe.

2.2 COLD ON-COLUMN INJECTOR.

When using an on-column injector the column is extended into the inlet and the sample is injected directly onto the column head. The subsequent heating of the column and the flow of the carrier gas facilitate the vaporisation of the analyte. The column is extended into a heated rotary valve, with an open and shut lever. A very fine syringe needle passes through the valve and the sample is injected onto the top 2 cm of the column. The top of the column is cooled (nitrogen gas) for 30 seconds prior to the injection. Direct application

of the sample means that the on-column injection is very suitable for trace analysis, with the amount of analyte applied to the column maximised. When used to analyse biological samples, the top few centimetres of the column must be removed occasionally to prevent accumulation of contamination.

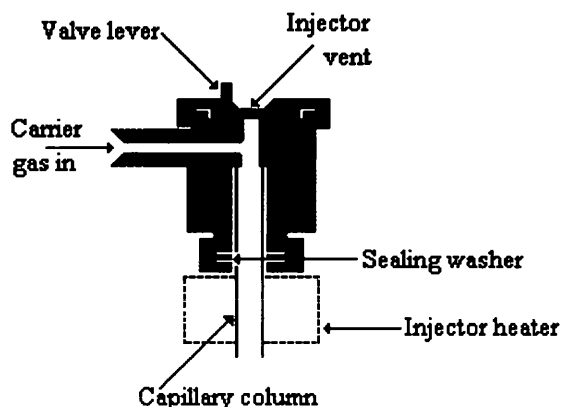


Figure 2.1 Schematic of an on-column injector

2.3 MASS SPECTROMETRIC DETECTORS.

In this project where gas chromatographic analysis was followed by mass spectrometric detection, two forms of mass spectrometer were used: quadrupole and ion trap systems. The first mass spectrometer used was a VG20-250 quadrupole mass spectrometer coupled to a Hewlett Packard GC. The quadrupole device (as illustrated in Figure 2.2) consists of molybdenum rods (4 x 12 mm) precisely aligned by two ceramic discs and screws. Electrical power of up to 12 kV peak to peak was applied to the rods and a radio-frequency potential is superimposed. Ions are repelled from the ion source into the quadrupole analyser with a small accelerating voltage. From the application of these fields oscillations of ions occur, some leading to stable trajectories when the ions will be transmitted to the detector. Those with unstable trajectories eventually collide with the rods and do not enter the detector. The ion detector in the case of these analyses was a Galileo 4771 series

photomultiplier. The multiplier has a conversion dynode operating at 0 volts in the positive mode and +5 kV in the negative ion mode. The upper limit of the source pressure is about 1.05×10^{-5} Pa and for the quadrupole and the detector is approximately 1.05×10^{-6} Pa.

The EI/CI ion source of the quadrupole mass spectrometer consists of a gas-tight ion chamber, which has alternative ion exit apertures, selected from outside the source housing. The larger is used when EI spectra are acquired, the smaller allows a higher chamber pressure for CI. Large mass filters and pre-filters allow detection of ions up to 2000 mu.

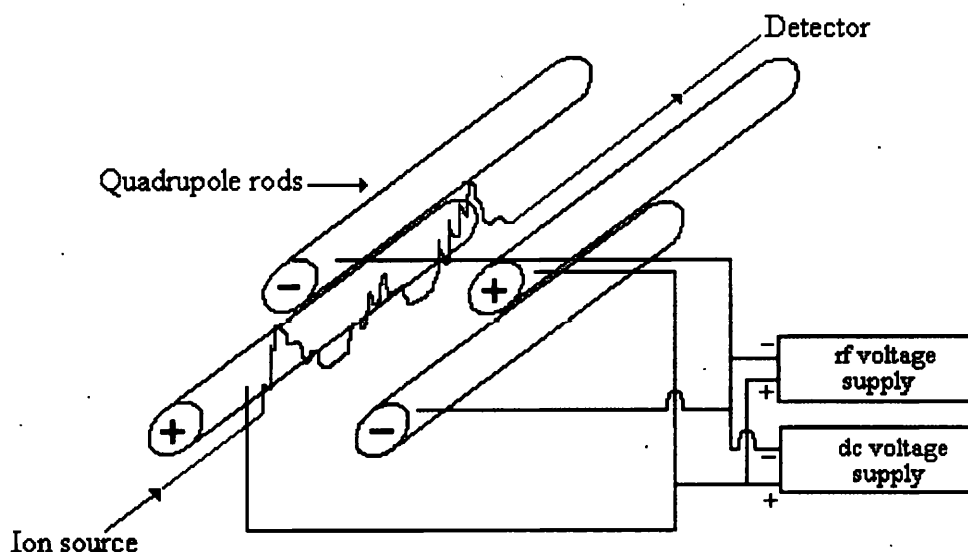


Figure 2.2 Schematic of a quadrupole mass spectrometer

In the second case an ion trap mass spectrometer was employed (Finnigan MAT ITD 800A). Ion Trap mass spectrometers work on the principle of the ions being trapped in an evacuated cavity, through the application of appropriate electric fields. The trapped ions are then expelled from the trapping cavity selectively by mass. When represented schematically, the trap can be compared to a two dimensional slice through a quadrupole,

with electrodes top and bottom (end caps) of the cavity and a central ring electrode, this structure is illustrated in Figure 2.3.

The end electrodes are in this case earthed, though they can be either of ac or dc bias, while the ring electrode has a sinusoidal radiofrequency potential applied to it. Application of differing potentials or earthing of these end caps can be utilised to alter the mode of the ion trap to include the formation of a burst of ions by passing a pulse of electrons into the trap. Other modes include the trapping of all created ions and by changing the Rf potential, thus creating unstable motions, and sequentially ejecting according to m/z values (smaller m/z values first).

Ions are therefore trapped within the chosen electrical field and those with stable trajectories in this oscillating field will remain in the cavity while others will be ejected. Ions in the trap must acquire unstable motion before they can be released from the trap. On ejection from the cavity many ions leave through the lower end cap and strike the detector and are recorded. This very rapid cycle is then repeated on another batch of ions, thus building up mass spectra. Chemical ionisation with isobutane is also possible and was used within this project. Ion traps are operated with approximately 0.13 Pa of trapped helium. This has the effect of improving both the resolution and the sensitivity of the instrument.

Collisions of the reagent ions with helium tend to calm the amplitude and velocity of their movement forming a more central and tighter bunch of ions for release. This improvement can be seen in the corresponding increase in peak heights and their narrowing.

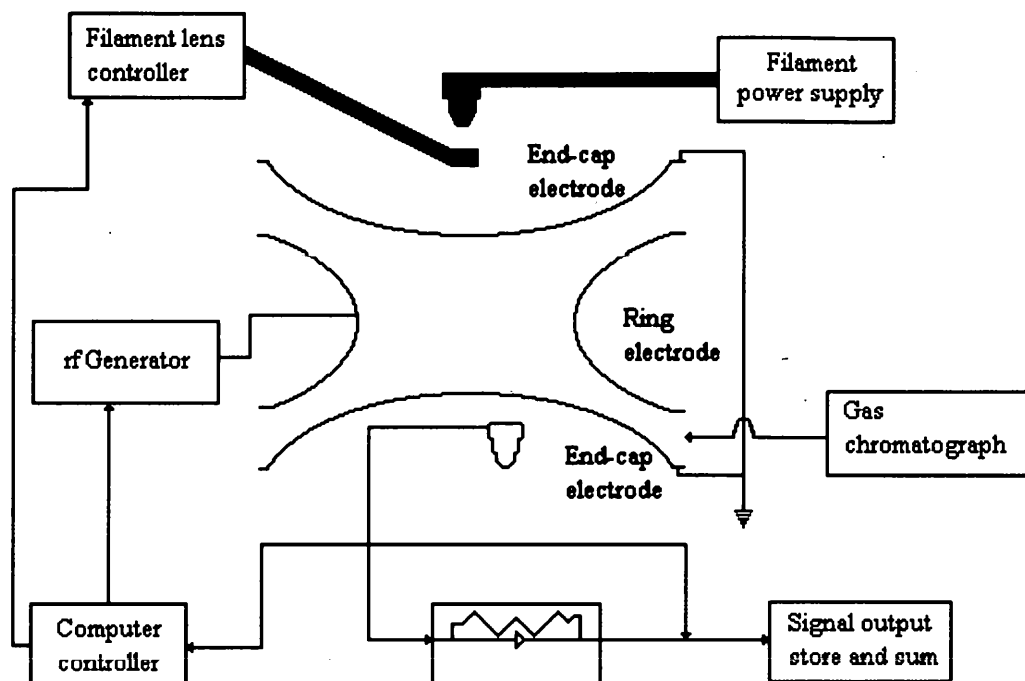


Figure 2.3 Schematic of the ion trap detector

The different systems used for the analyses outlined in this thesis were:

(i) A VG 20-250 quadrupole system coupled to a Hewlett-Packard 5890 fused-silica capillary column, 25 m x 0.22 mm I.D. and 0.25 μm film thickness, and an SGE OCI-3 on-column injector. Helium (35 cm/s linear velocity) was used as the carrier gas. On-column injections were made at an initial column temperature of 50 $^{\circ}\text{C}$. The temperature was immediately ramped at 30 $^{\circ}\text{C}/\text{min}$ to 80 $^{\circ}\text{C}$ and then increased to 250 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}/\text{min}$ and held for 15 min. The direct line interface was maintained at 250 $^{\circ}\text{C}$ and a source temperature of 200 $^{\circ}\text{C}$ was used. EI was effected with an electron beam energy of 70 V and emission current of 100 μA .

(ii) A Finnigan MAT bench-top ion trap detector, Model 800A, coupled with a Varian series 3400 gas chromatograph fitted with the same column as in (i). The GC conditions were as above except that splitless injection at 230 $^{\circ}\text{C}$ was used and the initial column

temperature was 90 °C. The open-split interface was maintained at 240 °C. The electron beam energy of 70 eV and emission current of 5 μ A. The ion source temperatures were 220 °C (for EI) and 180 °C (for CI). Chemical ionization mass spectra were recorded using isobutane as a reactant gas.

In both GC/MS systems mass spectra were recorded repetitively every second.

2.4 INFRARED SPECTROSCOPY (IR).

The infrared region is on the lower frequency side of the visible region of an electromagnetic spectrum, where energy level separations are smaller. In IR spectroscopy the sample is irradiated in the infrared frequency range and individual functional groups absorb at different frequencies within this range. Downward bands typical of a particular functionality can then yield useful structural information about a compound of interest.

2.5 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR) .

In this technique a magnetic field is applied to the sample of interest which causes the nuclear spin states to align with or against the force of the applied field. Nuclei such as hydrogen and carbon thirteen (^{13}C , the isotope of normal ^{12}C) then absorb in the radiowave region of the electromagnetic spectrum. A number of nuclei can be observed through the use of magnetic fields to provide information not only about themselves but also about their interactions with neighbouring nuclei. With the combined information of ^1H and ^{13}C spectra much structural information has been gained during the course of this work.

NMR spectra were obtained on a 90 MHz Jeol FX90Q multinuclear spectrometer and a 400 MHz Brücker JNM-EX400 instrument.

2.6 FAST ATOM BOMBARDMENT/MASS SPECTROMETRY (FAB/MS).

Fast atom bombardment spectra were obtained on the VG 20-250 quadrupole system used for GC/MS described above (2.3 [i]) The gas used to generate the fast atoms was xenon at a cylinder pressure of 6.895×10^4 Pa. This pressure is adjusted to give a gun current of 1 mA at about 8 kV. The atom beam emerging from the source is largely concentrated about an axis 2 mm in diameter. Glycerol (2-5 μ l), the matrix solvent, was placed on the stainless steel tip to which the samples, in solid form, were added and dissolved. Spectra were obtained every second, alternating between the positive and negative ion modes of acquisition.

2.7 ELECTROSPRAY MASS SPECTROMETRY (ESI/MS).

Electrospray mass spectrometric analyses of samples of biological origin were performed on two systems: (i) A VG Trio-2000 operating with a cone voltage of about 30 V. The carrier solution was water/acetonitrile (50:50) containing 1 % formic acid at a flow rate of 5 μ l/min. A 10 μ l injection loop was used for sample introduction. (ii) A VG Platform, bench-top, single quadrupole mass spectrometer (Fisons Instruments/VG BioTech). Loop injections were performed using a Rheodyne 8125 injector with a 5 μ l loop. Injection volume was 5 μ l from the sample residue in 50 μ l acetonitrile/water. The carrier solution and flow rate was as in (i). This technique will be discussed further in Chapter 6 of this thesis. The electrospray work completed for this thesis was carried out at VG biotech (now Micromass), Altringham, Cheshire, UK.

2.8 LIQUID CHROMATOGRAPHY-ELECTROSPRAY MASS SPECTROMETRY (LC-ESI/MS).

LC/MS was performed using two columns: (i) an Applied Biosystems, Aquapore RP-30, 100 x 1.0 mm column. Gradient elution at 40 μ l/min was performed using a linear gradient from 95% 0.01M ammonium acetate, 5% methanol to 100% methanol over 20 min with the eluant introduced directly to the electrospray source. Analyses were also carried out using (ii) a Phase Separation 1 mm x 25 cm C8 column with acylcarnitines isocratically eluted with 100% 0.01M ammonium acetate for 5 min followed by a ramp to 100% methanol, with introduction into the source as above. This technique will be discussed further in Chapter 6 of this thesis.

CHAPTER 3

THE ANALYSIS OF CLINICAL URINE SAMPLES.

3.1 INTRODUCTION.

A study of acylcarnitines in a manner similar to that described in this thesis has been in progress at the Open University for three years prior to the initiation of this project. The initial developmental work was carried out towards the analysis of mono-carboxylic acylcarnitines from spiked and clinical urine samples and was successful in the diagnosis of a small number of inherited metabolic diseases from their acylcarnitine profile by GC and/or GC/EIMS.

The initial investigation into the viability of GC/MS for the analysis of acylcarnitines led also to the development of a work-up which was carried out on 0.5 ml urine samples prior to analysis (see Section 3.4.2) [1]. This work-up has been modified, giving rise to a second method of sample preparation which, being a more simple solvent extraction [2], can now be used in place of the former. This modification was developed due to evidence of inefficient extraction of long-chain acylcarnitines from urine prepared via the former ion-exchange procedure. The poor recovery via ion-exchange chromatography was thought to be due to irreversible binding of the long-chain acylcarnitines to the resin. The modified method involves an alcohol extraction using the guideline of like-dissolves-like (i.e. long-chain alcohols extract long-chain acylcarnitines more efficiently) followed by centrifugation to breakdown any emulsion formed, removal of the supernatant and drying of the solvent layer. Both solvent extraction and ion-exchange chromatography leave a residue, which contains acylcarnitines in a form that is sufficiently pure for subsequent chromatographic analysis.

Acylcarnitines themselves are not suitable for gas chromatography, due to their zwitterionic non-volatile nature. Sample volatility is a requirement of the technique. To

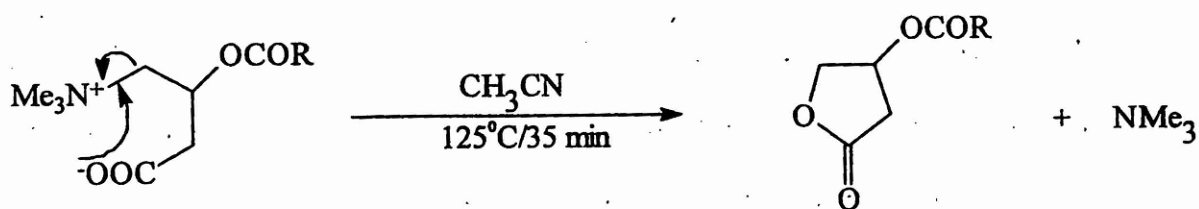


Figure 3.1 Cyclization of acylcarnitines to form lactones

overcome this problem, chemical derivatization through a cyclization method was developed (see Figure 3.1). This procedure involves heating the extracted acylcarnitines in acetonitrile in the presence of a small amount of base for about 35 minutes as described in Section 3.5.3. Under these conditions the carboxylate functionality intramolecularly attacks the quaternary ammonium group. Nucleophilic attack displaces trimethylamine yielding a lactone. This lactone ring derivative is volatile in nature and suitable for analysis by gas chromatography. An added advantage of this derivatization is that the lactone ring retains a clear memory of the acylcarnitine from which it was formed, unlike any degradative procedure that converts the acyl groups into the corresponding carboxylic acid (as illustrated in Figure 3.1).

The work described in this chapter includes the isolation and analysis of a number of clinical urine samples for the presence of diagnostic acylcarnitines. In the cases illustrated, acylcarnitines were isolated from urine using the established ion-exchange method of Lowes and Rose (1990) [1]. Both urine samples presented were worked-up and derivatized approximately two years before the following set of analyses were carried out, which is a testament to the stability of the lactone structures formed from acylcarnitines extracted from biological fluids. These examples demonstrate (i) the variety of acylcarnitines which can be extracted, derivatized and detected via this method, (ii) studies of both endogenous and exogenous origin, (iii) application of an ion trap detector for the first time and (iv) the development and advantages of chemical ionisation.

3.2 DIAGNOSIS OF AN INHERITED METABOLIC DISEASE.

3.2.1 INTRODUCTION TO THE CLINICAL PROBLEM.

A urine sample was presented from the patient, a neonate, diagnosed as suffering from the inherited metabolic disease medium-chain acyl-CoA dehydrogenase deficiency (MCADD).

The sample was prepared (Section 3.5.2 - 3) and analysis was carried out by GC/MS using a VG 20-250 mass spectrometer with a Hewlett Packard GC (for analysis conditions see Section 2.3(i)). The data obtained did not fully agree with the proposed diagnosis of MCADD. These results were such that the diagnosis, which at the time had become a cause of concern to the clinicians involved, was refined to multiple acyl-CoA dehydrogenation deficiency (MADD), also known as glutaric acidemia type III (GA III). This change in diagnosis has been confirmed by independent medical analysis.

This male patient was born in 1984 and from the age of one year had suffered episodes of hypoketotic seizures, hypoglycaemia (when fasting for >15 hrs) and moderate muscle weakness. In 1988 the patient presented with Reye-like symptoms including hepatic dysfunction, hyperammonemia, hypoglycaemia (without ketosis) and moderate metabolic acidosis. Urine analysis showed massive dicarboxylic aciduria and increased levels of organic acids and elevated levels of organic acids. Elevated levels of medium-chain fatty acids (including cis-4-decanoic acid) were found in plasma.

However, the sensitivity of the original analysis was very poor and only the two major disease specific acylcarnitines were identified. The sample was examined again with an ion trap detector and chemical ionization also applied. Before the analysis is discussed, an introduction to the disease in question will be outlined.

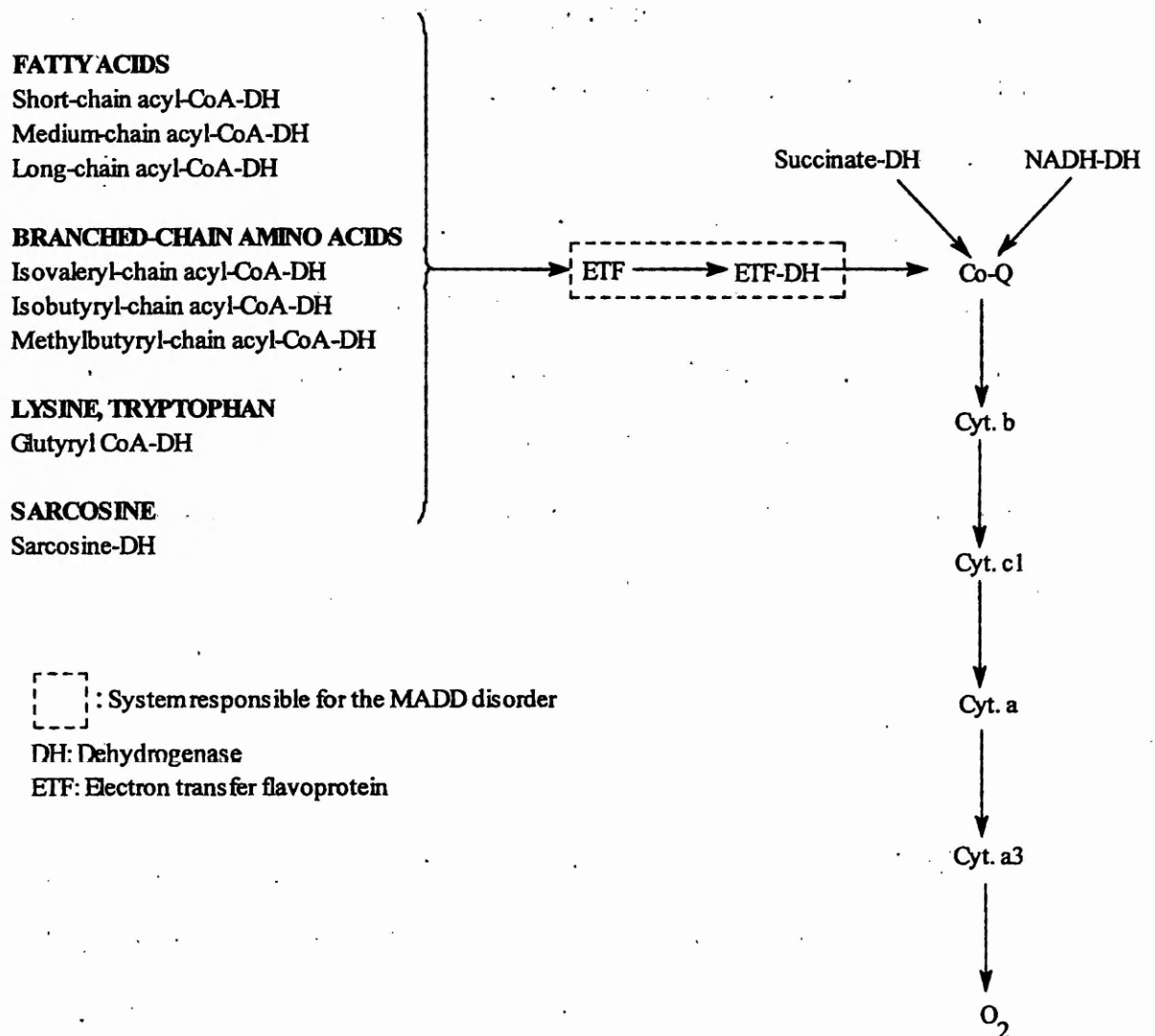


Figure 3.2 Metabolic system and enzymes causing the disorder MADD. This disorder encompasses a complex group of co-factors powering the enzyme system of dehydrogenation and characterized by multiple acylcarnitines, and other indicators, in biological fluids.

The MADD disease is different in nature from metabolic defects usually identified and those described elsewhere in this thesis. It is a disorder encompassing a complex group of co-factors (i.e. ATP, ETF) which power the enzyme system of dehydrogenation. It is characterised by disturbances to the mitochondrial flavin-containing acyl-CoA dehydrogenase enzymes as noted above. These enzymes are mitochondrial dehydrogenases, which require flavin adenine dinucleotide (FAD), as a co-factor [3] and oscillate between oxidised, two-electron-reduced, and one-electron-reduced states [4]. Inherited metabolic diseases of β -oxidation are usually characterized by a deficiency or abnormality of a specific chain-length acyl-CoA dehydrogenase within β -oxidation. While showing these characteristics, MADD also presents itself as disorders of branched-chain amino acid catabolism with specific reference to isovaleryl-CoA and α -methylbutyryl CoA dehydrogenases, the enzymes glutaryl-CoA and sarcosine-CoA dehydrogenases are also affected [5]. The enzyme systems affected by the metabolic disorder MADD are outlined in Figure 3.2

Patients with MADD fall into a number of distinct groups. The autosomally recessive nature of the disorder means that each type is consistently found within a family. In one family however, X-linked inheritance has been proposed with the disease manifesting itself in neonates [6].

Three types of MADD have been described (i) neonatal onset without congenital anomalies, (ii) neonatal onset with congenital anomalies and (iii) mild or later onset. Lethargy, hypotonia, severe acidosis and hypoglycaemia usually characterise the first and second forms. Infants without congenital anomalies present symptoms within the first twenty four hours and seldom survive the first week of life, this is referred to as the Type 1 disease. In the second neonatal form (MADD Type 2) a lack or absence of riboflavin may

be responsible for dysmorphic characteristics observed [7, 8, 9, and 10]. Neonatal patients with congenital anomalies are often premature and present with symptoms, as described above. Also, an odour similar to that present in isovaleric acidemia, due to an accumulation of volatile short-chain organic acids, occurs within the first few days of life. The third MADD type, the milder, late on-set form of the disease is manifested in similar clinical symptoms as the former but the patient will probably only suffer intermittent illness [11]. Patients with this type of MADD have very variable characteristics and times of presentation. Some patients have been symptom-free during childhood, presenting with symptoms only in adult life [12]. In these cases the disorder may be treated with riboflavin administration [13, 14]. Patients who only later present with MADD symptoms may develop a Reye Syndrome-like illness and these patients survive longer [15, 16, 17, 18]. Studies at the protein level in MADD have suggested a deficiency in the electron transfer flavo-proteins (ETF) or electron transfer flavo-protein ubiquinone: oxido-reductase (ETF-DH) as potential factors in this disorder [19, 20, and 21].

Urinary organic acids have been reported as abnormally high in many cases of MADD indicating the presence of short-chain volatile acids and a wide range of other acid types. The excretion of compounds of this chain-length is due to a defect in or an absence of the enzyme medium-chain acyl-CoA dehydrogenase within β -oxidation, preventing oxidation beyond this point. MCADD can be incorrectly diagnosed as Reye's syndrome or sudden infant death syndrome (SIDS). MCADD is triggered by excessive fasting (> 12 hr) and in some cases is only identified when a sibling is identified as having suffered from the disorder, post mortem. Also characteristic of this disorder is a secondary carnitine deficiency and dicarboxylic aciduria, with the excretion of $C_6 - C_{10}$ dicarboxylic acids, when stressed from fasting. Organic acids are also reported as elevated in serum and cerebrospinal fluids.

The acylcarnitines detected in urine samples of MADD would also be expected to encompass a wide range of acyl groups though those in plasma may be of lower concentration [22]. The diversity of acylcarnitines expected in the case of MADD however, allows its unique diagnosis and this variety will be clearly illustrated with results from two clinical urine samples analysed. The analytical data obtained are discussed in this thesis chapter.

3.2.2 INITIAL SAMPLE ANALYSIS

Prior to the work presented in this thesis the urine sample, prepared as in Section 3.4.2, and described above was analysed by GC/EIMS using a VG 20-250 mass spectrometer (Chapter 2, Section 2.3). The results obtained indicated the presence of only two diagnostic acylcarnitines, at levels close to the detection limit of the instrument, and the internal standard [25]. The internal standard used in this study was 4-phenylbutanoylcarnitine which whilst mimicking the actions of acylcarnitine is a synthetic rather than a naturally occurring acylcarnitine. EI and CI spectra for the internal standard are shown in Appendix (i).

The two urinary acylcarnitines were identified and confirmed as their corresponding lactones through their gas chromatographic retention times and mass spectral information, as compared with those from synthetic standard lactones of octanoylcarnitine and isovalerylcarnitine. The presence of these two metabolites in a clinical urine sample was the evidence that gave rise to the diagnosis of MADD as isovalerylcarnitine would not be expected in a simple case of MCADD. As described above (Section 3.2.1) these two metabolites in a single urine sample would be indicative of a defect within β -oxidation and of isovaleryl-CoA dehydrogenase, which is involved in leucine catabolism.

The detection of octanoylcarnitine lactone is indicative of the disease MCADD, characterised by the presence of medium chain acylcarnitines (C₆ - C₁₀) in biological fluids. The mechanism for secondary carnitine deficiency in MCADD is unknown but is possibly due to the high levels of excreted octanoylcarnitine [48].

The presence of the isovalerylcarnitine lactone on its own would be evidence for the disease isovaleric acidemia. Isovaleric acidemia is a defect of short, branched-chain fatty acid oxidation, which was the first disorder of fatty acid metabolism identified by GC/MS, in 1966 [23]. At that time the enzyme involved in this defect, isovaleryl-CoA dehydrogenase, had not been discovered [24]. This acidemia is characterised by the presence of isovaleric acid in the blood, plasma or the urine and often presents itself in the form of recurrent episodes of lethargy and vomiting. An infection and/or large intake of protein often trigger this, with treatment in these cases by glucose infusion. An odour of sweaty feet is also characteristic of this disorder, caused by an excess of isovaleric acid. Plasma analysis by GC for these short-chain fatty acids during an acute episode shows a level of isovaleric acid which is several hundred times above the level in controls, even at a basal level in patients the acid level is several times higher than that of controls. Small amounts of hyperammonia and hypoglycaemia are indicative of this disorder as is secondary carnitine deficiency [24].

3.2.3 RESULTS AND DISCUSSION.

Further work has now been carried out on the urine sample described above, after approximately two years of storage at about 0° C, in a Teflon sealed sample tube. These results were obtained using a Varian GC with a DB5 column connected to a Finnigan MAT ITD 800A ion trap system. The sample was run over a temperature programme (for analysis conditions see Section 2.3). Table 3.1 provides a listing of acylcarnitines

identified from the urine sample, and confirmed by retention times, mass spectra and library matches with synthesised or commercially obtained standards. Given that the mass spectra were acquired every second with no interscan delay, the scan number corresponds to the retention time in seconds. All of the lactones with butanoyl or larger acyl groups exhibit ions at m/z 85 (7) and 144 (6), and lower homologues yield ions at m/z 84 (5). The process of fragmentation of acylcarnitines to yield these very characteristic fragments is illustrated in Figure 1.5, Chapter 1, opposite page 38.

Identification of acetylcarnitine lactone was made in the EI mode through the occurrence of the protonated molecule, m/z 145 (20%) which is indicative of some self-chemical ionization in the ion trap when large amounts of a compound are present. Such self-chemical ionization is common in an ion trap when high concentrations of acylcarnitine elute. The base peak in the EI spectrum of acetylcarnitine at m/z 84 (100%) is due to elimination of acetic acid, giving the unsaturated lactone ring fragment (5). In the larger homologues the fragmentation to give the response at m/z 144 is a McLafferty rearrangement of the ring and the beginning of the side-chain (6). The rearrangement is characteristic of acylcarnitines beginning at butanoylcarnitine. Fragmentation of all acylcarnitines produces m/z 85, which is derived from the lactone ring (7) and is often the base peak with EI analysis.

Chemical ionization of lactones, using isobutane, is characterized by three particular m/z values. All acylcarnitines produce m/z 85, which is again derived from the lactone ring (7), and m/z 125 which has not been structurally identified. The third (and usually base) peak identified in spectra of these compounds is the protonated molecule, at an m/z value one greater than the relative molecular mass of the acylcarnitine in question.

Chromatogram A:PH(1)
 Comment: DIVRY URINE SAMPLE INJ 230 90-250C 10C/MIN MADD
 Scan Range: 200 - 499 Scan: 200 Int = 789 @ 3:21 100% = 16962

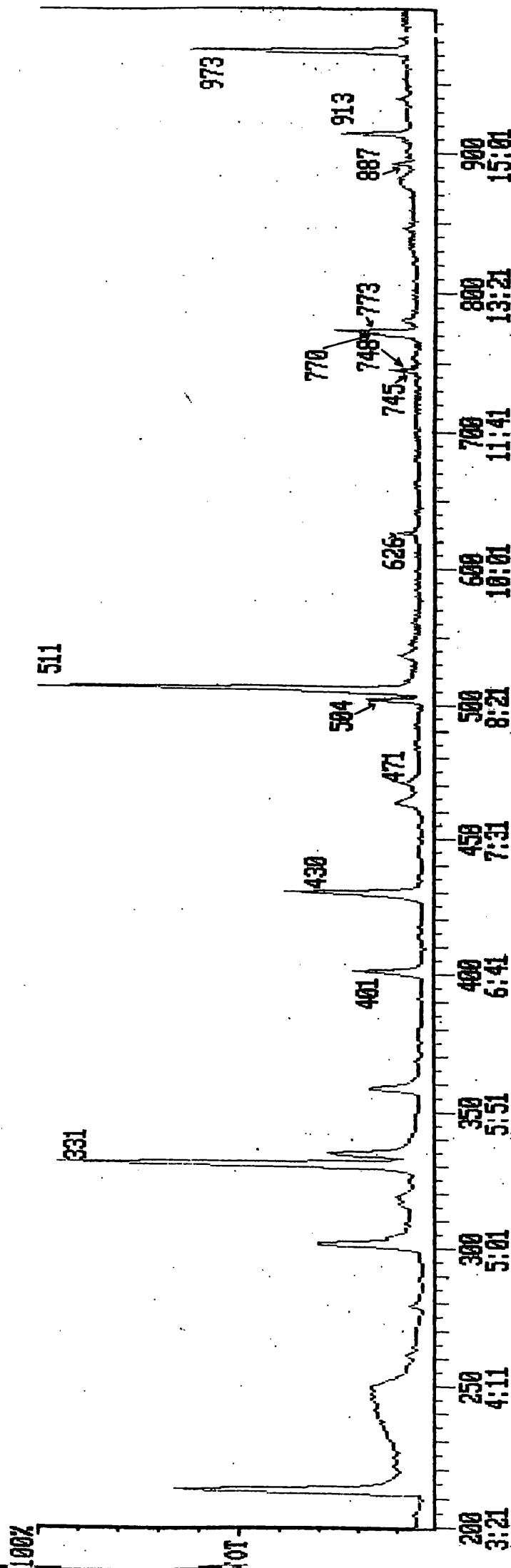


Figure 3.3 Illustrates a typical GC/CIMS total ion chromatogram for a MADD urine sample. Table 3.1 lists the acyloxylactones identified

from this chromatogram and provides a retention time (by scan number) comparison to standard acyloxylactones (where the data is available). A selection of EI and CI mass spectra generated from the sample analysis shown are provided as Appendix A.

Figure 3.3 illustrates a typical GC/CIMS total ion chromatogram for a MADD urine sample, which has been prepared for analysis in the manner described in this chapter. As can be seen there are a number of significant peaks, which may prove diagnostic (these acyloxylactones are listed in order of chromatographic retention time in Table 3.1). A selection of mass spectra used to identify acylcarnitines from the MADD sample and compile Table 3.1 are presented in Appendix A 1-9.

TABLE 3.1. Comparison of GC retention times: standard lactones vs. scan number of peaks as shown in Figure 3.3.

COMPOUND NAME	SCAN NO./STANDARD	SCAN NO./SAMPLE
(a) Acetylcarnitine lactone	329	331
(b) Propanoylcarnitine lactone	400	401
(c) Isobutanoylcarnitine lactone	431	430
(d) Butanoylcarnitine lactone	472	471
(e) 2-Methylbutanoylcarnitine lactone	501	503
(f) Isovalerylcarnitine lactone	509	511
(g) Hexanoylcarnitine lactone	624	626
(h) An octenoylcarnitine lactone	-	745
(i) An octenoylcarnitine lactone	-	748
(j) Trans-Oct-3-enoylcarnitine lactone	766	-
(k) An octenoylcarnitine lactone	-	770
(l) Octanoylcarnitine lactone	772	773
(m) A decenoylcarnitine lactone	887	887
(n) Decanoylcarnitine lactone	908	913
(o) 4-Phenylbutanoylcarnitine (IS)	969	973

Acetylcarnitine lactone (Figure 3.3 scan 331 and Appendix A1) was clearly shown to be present in the urine sample which has been diagnosed as that of a patient suffering from the disorder, multiple acylcarnitine dehydrogenation deficiency. This compound is the end product of β -oxidation (and has a number of other sources). This ambiguous source of acetylcarnitine means that the presence of this compound is not diagnostic.

The second acylcarnitine identified from the gas chromatogram was propanoylcarnitine lactone, the C₃ side-chain acylcarnitine (Figure 3.3 scan 401 and Appendix A2). The presence of this acylcarnitine is in itself indicative of a number of inherited disorders of fatty acid or amino acid metabolism, these are discussed further in Chapter 4 of this thesis. Propanoylcarnitine lactone was identified with acetylcarnitine initially by the presence of the characteristic fragment in the EI mode at m/z 84 (30%), in this case the fragmentation yielding m/z 57 (100%) is also significant, probably resulting from $\text{CH}_3\text{CH}_2\text{CO}^+$. A protonated molecule resulting from self-chemical ionization in the EI mode is seen here at m/z 159 (5%). In the CI spectrum the base peak is m/z 85 with the characteristic m/z 125 (5%) also present. With a relative molecular mass of 158 the compound is detected with an $[\text{M} + \text{H}]^+$ ion at m/z 159 (30%).

Separation of isomeric acylcarnitines has also been shown in a number of cases from this MADD urine sample. Isobutanoylcarnitine (Figure 3.3 scan 430 and Appendix A3) and butanoylcarnitine (Figure 3.3 scan 471) lactones both have a relative molecular mass of 172 and these two compounds are separated by approximately forty seconds. The EI mass spectrum of isobutanoylcarnitine lactone indicates the expected fragmentation to yield m/z 84 (80%). The base peak at m/z 71 is probably the side-chain ion, $\text{C}_3\text{H}_7\text{CO}^+$. Again self-chemical ionization of this compound is apparent with the detection of a peak at m/z 173 (10%). In the CI spectrum the fragmentation pattern is now familiar with responses at m/z values of 85 (100%), 125 (5%) and the protonated molecule at m/z 173 (65%). The mass spectrum of butanoylcarnitine lactone was as expected very similar to that of its isomer but with a larger peak at m/z 144.

The next pair of peaks in the chromatogram from this MADD urine sample are also isomers. 2-Methylbutanoylcarnitine lactone (Figure 3.3 scan 504 and Appendix A4) and

isovalerylcarnitine lactone (3-methylbutanoylcarnitine lactone, as shown in Figure 3.3 scan 511 and Appendix A5) are separated by eight seconds in retention time. While this is not as significant as the previous separation it can be seen that the peaks are resolved. The EI spectra are very similar though the relative abundance of individual fragment ions, such as m/z 144, is higher in the case of isovalerylcarnitine lactone. The corresponding CI spectra are also similar, with base peaks at m/z 187 for $[M + H]^+$ ions, with differences only in abundance and presence or absence of smaller fragments.

Those acylcarnitines described above are all short-chain acylcarnitines and are from various sources within the metabolic system. A second chain-length group of acylcarnitines was also detected, these are medium-chain acylcarnitines (C_6 - C_{10}). The first of these is hexanoylcarnitine lactone (Figure 3.3 scan 626, Appendix A6), the six carbon side-chain acyloxylactone. The EI spectrum of hexanoylcarnitine lactone shows a response at m/z 201 (2%) indicating some self-chemical ionization of the sample and the target fragments of m/z 85 (100%) and 144 (20%) are also significant. In the CI mode the mass spectrum the base peak corresponds to the lactone ring fragmentation and m/z 125 can also be detected with a relative ion abundance of about 5% that of the base peak. The protonated molecule of hexanoylcarnitine at m/z 201 (50%) is also significant.

Unsaturated C_8 acylcarnitines were also detected in this sample. Four potential octenoylcarnitines are featured in Table 3.1, an example of this group is presented as Figure 3.3 scan 745, Appendix A7). Although these only differ in the position of the double bond, all having a relative molecular mass of 226, their separation is significant enough to determine their presence using mass chromatograms for the m/z 85 fragment or the protonated molecule m/z 227 in the CI mode. In both the EI and CI modes the characteristic fragments described above for the saturated acylcarnitines are also present.

The saturated C₈ acylcarnitine, octanoylcarnitine lactone (Figure 3.3 scan 773, Appendix A8), was also detected in this sample. In the EI mass spectrum octanoylcarnitine lactone illustrates typical acylcarnitine fragmentation, m/z 85 (100%), 144 (15%) and some self-chemical ionization to yield an $[M + H]^+$ at m/z 229 (1%). The CI spectrum is also as expected with m/z 85 (100%), 125 (5%) and 229 (65%). In the case of the C₁₀ acylcarnitines an unsaturated, decenoylcarnitine lactone (Figure 3.3 scan 887), was identified and the saturated decanoylcarnitine lactone (Figure 3.3 scan 913) was also identified. The internal standard used in this extraction is also included as Figure 3 scan 973, Appendix A9.

In the cases of all the acylcarnitine lactones described, identification was based on both retention time and mass spectral information. From Table 3.1 it can be seen that the retention times of the acylcarnitine lactones detected in this urine sample correspond very closely (within 4 seconds) to those of the standards and this was found to be the case both for within-day and between day analyses.

The ion trap spectra of standard acylcarnitine lactones were used in the form of a computerised library and sample acylcarnitine lactones matched well with their equivalent standard spectra. In general the retention times for acylcarnitines have proved very reproducible, both on a day-to-day basis and in the longer term. This reproducibility was such (as illustrated in Table 3.1) that a comment on the presence or absence of a particular acylcarnitine lactone might be made on the basis of retention time prior to any mass spectral information.

There is no indication in this sample of the presence of any long-chain acylcarnitine lactones though this could be due to a number of factors including solubility and extraction

method. As this sample was prepared using ion-exchange columns it is likely that any long-chain acylcarnitines bound irreversibly to the cationic resin and would not be found in the final sample. This is one reason why the alcohol extraction method developed by Morrow and Rose might now be favoured [2].

The original GC/MS analysis of the same sample by Lowes [25] showed only two diagnostic acylcarnitines. The current detection of this wide range of acylcarnitines is due to improved sensitivity in the ion trap with respect to the quadrupole system. It might be considered that adoption of another extraction procedure could allow the detection of the long-chain acylcarnitines indicative of a long-chain Acyl-CoA dehydrogenase deficiency (LCADD) which would also be expected in the case of MADD. However, it should be noted that there was variability in response presented for a number of metabolites in recorded literature [11].

3.3. ANALYSIS OF BIOLOGICAL SAMPLES FOR DRUG METABOLITES.

3.3.1 THE METABOLISM OF SODIUM VALPROATE.

Valproic acid (VPA, 2-n-propylpentanoic acid) is an important anti-epileptic drug and is used for control of a range of epileptic seizures. Its pharmacological properties were first discovered in 1963 by Meunier *et al* [26]. Valproic acid is rapidly released in man in the form of the unchanged drug and there have also been a large number of metabolites reported in human and animal studies. The drug is a branched-chain fatty-acid and the identified metabolites indicate the use of a number of metabolic pathways as might be expected [27]. VPA can be conjugated with glucuronic acid to yield VPA-glucuronide, metabolized via β -oxidation, transformed by ω ₁-oxidation and metabolized by ω ₂-

oxidation (the products of these metabolic routes and their approximate plasma concentrations are shown in Table 3.2).

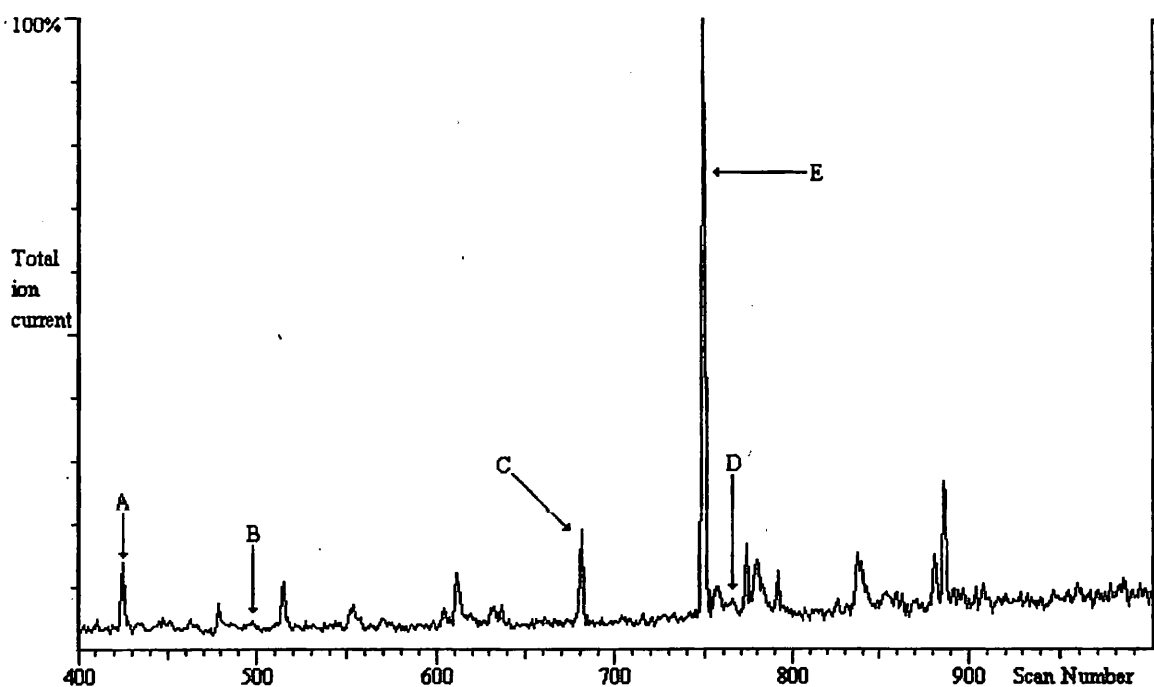
The presence of 4-keto-VPA has also been proposed as a product of valproate metabolism from human serum and urine extracts and its structure confirmed by GC/MS with synthetic reference compounds and the use of deuterium ($^2\text{H}_6$) labelled VPA [30]. VPA itself has been reported at 30-230 $\mu\text{g/ml}$ in plasma and is probably responsible for at least 80% of the anticonvulsant effect of VPA during chronic therapy in man [29].

Table 3.2. Known metabolites of the drug, valproic acid. [28, 29].

METABOLIC PATHWAY	METABOLITES (CONCENTRATION IN PLASMA)
β -oxidation	3-keto-VPA (1.5-14 $\mu\text{g/ml}$), with 2-en-VPA (4-18 $\mu\text{g/ml}$) and 3-hydroxy-VPA (0.3-7 $\mu\text{g/ml}$) as intermediates in this pathway.
ω 1-oxidation	2-n-propylglutaric acid (> 2 $\mu\text{g/ml}$), with 4-en-VPA (> 2 $\mu\text{g/ml}$) and 5-hydroxy-VPA (> 2 $\mu\text{g/ml}$) as intermediates.
ω 2-oxidation	3-en-VPA (> 2 $\mu\text{g/ml}$) and 4-hydroxy-VPA (> 2 $\mu\text{g/ml}$)

In treatment of patients with valproic acid the onset of the therapeutic effect of the drug is often longer than expected when compared with the short half-life of the drug [31, 32]. One possible explanation of this effect may be the slow accumulation of a valproate metabolite, which is then responsible for the pharmacological effect [28]. Carry-over effects of regular VPA treatment has been demonstrated in humans and patients up to two weeks after the drug was withdrawn, and VPA levels were below the detection limit in plasma [31]. No metabolite has been shown to be as active as VPA itself and observed metabolite concentrations in plasma suggest the anticonvulsant effect of the metabolites to

- (a) Neonatal patient, sodium valproate therapy,
Total ion chromatogram.



- (b) Neonatal patient, sodium valproate therapy,
Mass chromatogram, m/z 85.

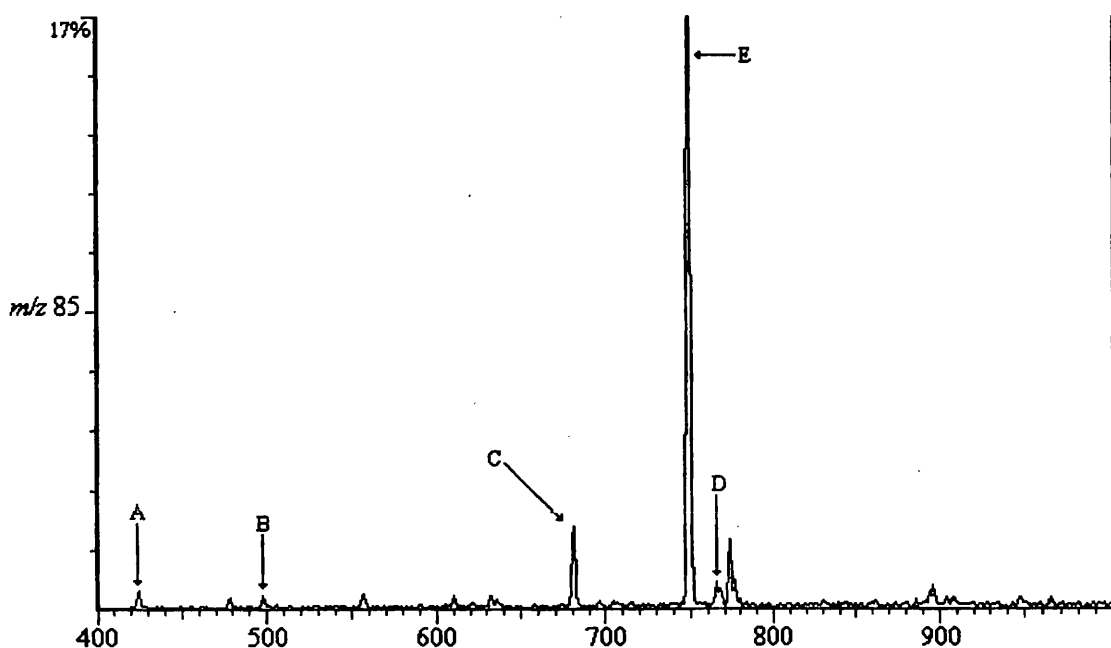


Figure 3.4 (a) TIC illustrating the acyloxylactones extracted from the urine sample of a neonatal patient undergoing Sodium Valproate therapy.
(b) Mass chromatogram, m/z 85, from the TIC above

Peak assignment - A 2-methylpropanoylcarnitine lactone, B 2-methylbutanoyl carnitine lactone, C valproylcarnitine lactone, D octanoylcarnitine lactone, peak E is discussed further in text and its EI and CI mass spectra are shown as Figure 3.5.

be 2-20% that of VPA with prolonged treatment in man thus questioning their therapeutic role [29].

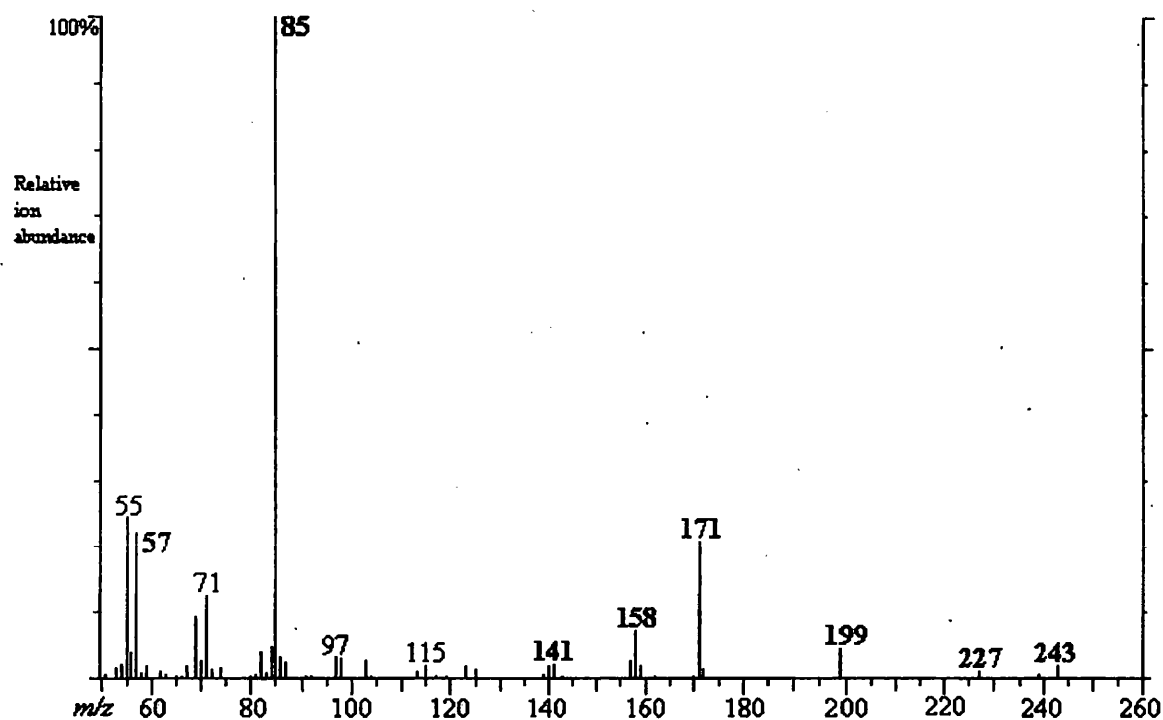
Schäfer et al (1980) verified that, after the glucuronide of valproic acid, 3-keto VPA is the main metabolite during long-term medication with valproic acid [33]. Löscher (1981) [28] demonstrated that the metabolic products of β -oxidation (including intermediates, see Table 3.2) were the main VPA metabolites in plasma

Valproic acid and its metabolites have also been shown to transfer across the placenta. Cord serum content of VPA was higher than in maternal serum with 3-keto-VPA and 2-en-VPA the main metabolites in both [34, 35]. VPA is thought to act, at least in part, via the GABA system in the brain with 4-en-VPA initially demonstrating potency similar to that of VPA in increasing brain GABA concentrations [29].

3.3.2 A CLINICAL STUDY

A urine sample from a patient treated with an anti-epileptic drug, sodium valproate, was prepared through the use of ion exchange column (Section 3.4.2) as a clean-up procedure, followed by derivatization [1]. Initial analysis using a VG 20-250 mass spectrometer with a Hewlett Packard GC indicated the presence of a number of compounds which were potential metabolites of this drug [25], though these were not confirmed. The stability of these acylcarnitines was demonstrated when two years after the initial data were obtained the sample was again analysed using a Varian GC with a DB5 column connected to a Finnigan MAT ITD 800A ion trap system. The sample was subjected to a temperature programme (see Chapter 2, Section 2.1 - 3 for GC and MS conditions).

(a) Neonatal patient, VPA treatment, EI mass spectrum



(b) Neonatal patient, VPA treatment, CI mass spectrum.

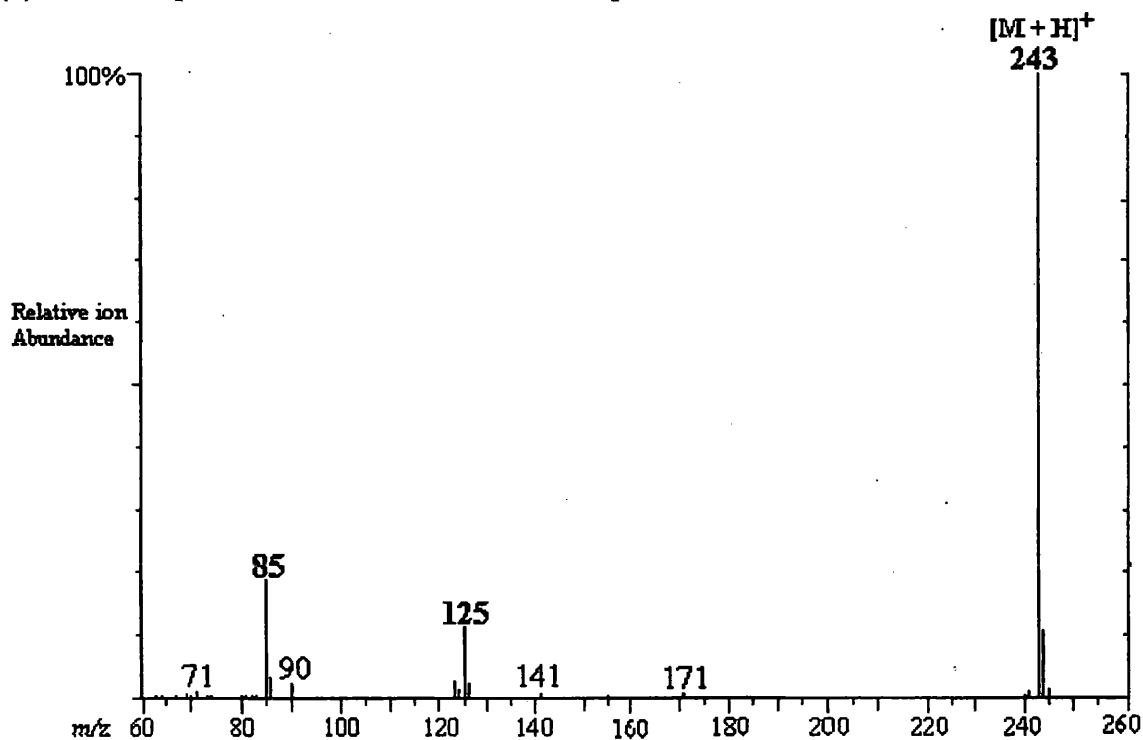


Figure 3.5 EI and CI mass spectra for peak E, from Figure 3.4.

These spectra were used to add weight to the proposed assignment of peak E as the lactone of 3-ketoalproylcarnitine, the proposed fragmentation of which is illustrated in Figure 3.6.

Focusing initially on the retention time window where one would expect to detect C₈ acylcarnitine lactones there are three peaks of interest in Figure 3.4, shown opposite the previous page. The first is consistent in retention time and fragmentation to the standard lactone derivative of valproylcarnitine (c) being dominated by $[M + H]^+$ ions at m/z 229 in the CI mode whilst in EI mode matching, within experimental error, the spectrum of standard material. The presence of a small amount of octanoylcarnitine lactone (d) was also detected and assigned by retention time and fragmentation pattern including a small response at m/z 229 in the CI spectrum. As can be seen from the mass chromatogram of the base peak, m/z 85, (Figure 3.4 (b)) the peak assigned to the isomers valproylcarnitine and octanoylcarnitine lactones, both having relative molecular weights of 228 to the nearest integer, are clearly separated. In Figure 3.4 there, however, is a third peak (e) in the retention time window of interest.

On the page opposite figure 3.5 shows the EI (a) and CI (b) mass spectra for the peak (e). The EI spectrum of this compound did not match that of any of the EI spectra present within our computer library of acylcarnitine lactones which includes most biologically active significant acylcarnitine lactones. The CI spectrum (b) is dominated by m/z 243 lending weight to an assignment of 242 as the relative molecular mass from the fragmentation pattern as described above. The presence of m/z 85 in both the EI and CI spectra is consistent with the base fragment of acyloxylactones as is the presence of m/z 125 in the CI spectrum. Referring again to the EI spectrum and looking at a pattern of fragmentation (Figure 3.6 overleaf) relevant to the CI assignment of 242 for the relative molecular mass a small peak at m/z 227 would be expected for $[M - CH_3]^+$. The peak present at m/z 243 might correspond to a small contribution of self-CI commonly giving rise to $[M + H]^+$ ions in the ion trap mass spectrometer. In the literature the main metabolite of valproic acid in man is reported to be the 2-propyl-3-keto ester of VPA (3-oxo-VPA) reported as 3 - 60% of the observed VPA metabolites as shown by mass

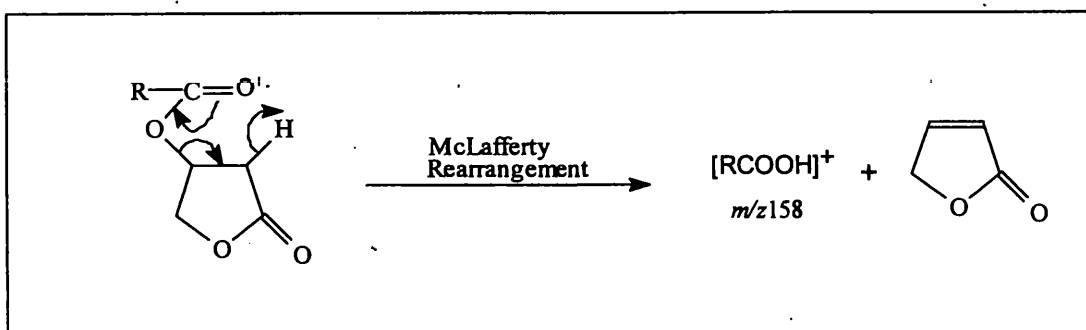
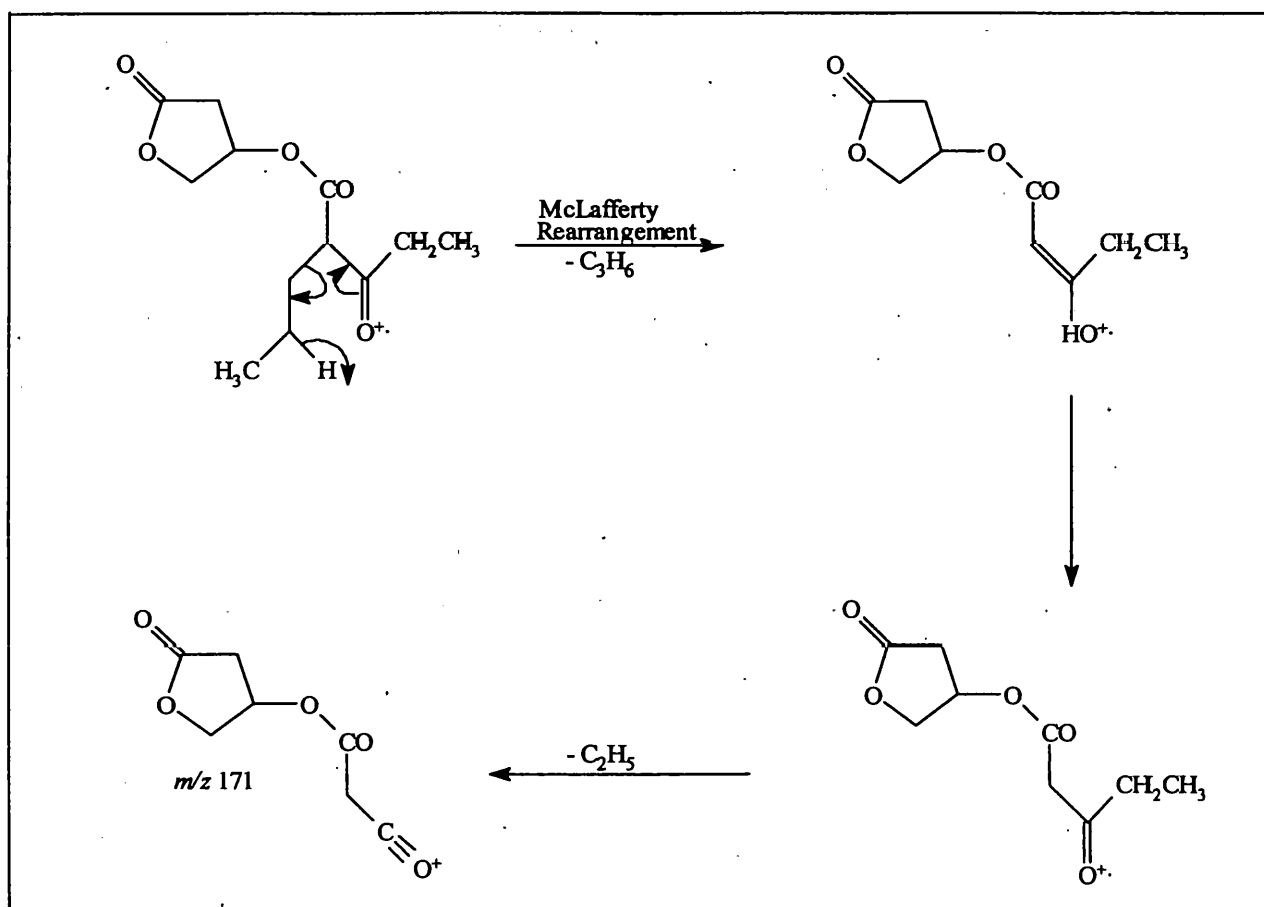
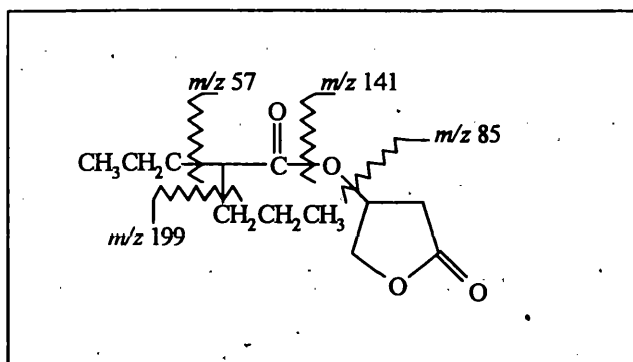


Figure 3.6 Structure and fragmentation of peak (E), proposed as the lactone derivative of 3-ketovaleryl carnitine. The EI and CI mass spectra of which are shown in Figure 3.5.

spectrometry and isotope labelling [36] in plasma [37, 38] and urine [39, 40, 41]. The EI mass spectrum of the methyl ester of 3-keto-valproylcarnitine has previously been reported [30]. The methyl ester showed three fragments in common with Figure 3.5 (a): loss of $\text{CH}_3\text{O}^\cdot$ to give m/z 141 (3%), formation of $\text{CH}_3\text{CH}_2\text{CO}^+$ at m/z 57 (100%), and a McLafferty rearrangement (as depicted in Figure 3.6) leading to m/z 171. These data add some weight to the CI interpretation of 242 as the relative molecular mass and the assignment of (e) as the lactone of 3-ketovalproylcarnitine. Confirmation of this assignment awaits synthesis of the proposed acylcarnitine. However, this is not a straightforward preparation as COCH_2COOH compounds decarboxylate very readily.

Propionic acid in urine of patients has been detected with its structure confirmed by mass spectrometry and chromatographic identity with synthetic reference compounds [37, 42] and valeric acid has been detected in rat urine [43]. In patients treated with VPA, equivalent acylcarnitines to these free acids might from the evidence above accumulate. The GC/CIMS chromatogram, as described above, was therefore studied for the presence of these short-chain acylcarnitines. A peak can be assigned to 2-methylpropanoylcarnitine lactone on the basis of its retention time compared to synthetic standards, its fragmentation showing m/z 85 and the protonated molecule ($[\text{M} + \text{H}]^+$) at m/z 159. This is labelled A in Figure 3.4 (opposite page 80). The presence of another acylcarnitine is also proposed, based on retention time compared to synthetic standards, fragmentation showing m/z 85 and 125 and the protonated molecule. This substance, marked as peak B, is ascribed to 2-methylbutanoylcarnitine lactone ($[\text{M} + \text{H}]^+$ at m/z 187) which is in agreement with previous findings [44].

3.4 CONCLUSIONS

What the data presented in this Chapter have clearly shown is that as the strength and sophistication of mass spectrometers increases so does the ability to use the instrumentation available to diagnose with greater accuracy and confidence.

The initial EI analysis of the first sample provided sufficient information to question the diagnosis of MCADD and to propose a diagnosis of MADD. The present application of a more sensitive analysis and CI in particular allowed a much more complete data set. Characteristic fragments have been illustrated and used to identify acylcarnitines from the TIC and to provide EI and/or CI mass spectra for representative acylcarnitine lactones as presented in Appendix A. Through the use of EI and CI we have been able to provide a range of data to allow the proposition of 3-ketovalproylcarnitine as a metabolite present from a patient being treated with valproic acid. For a set of compounds that provided few M^+ ions by EI, the confirmation of relative molecular mass via abundant $[M+H]^+$ ions in the CI mode facilitates structural assignment enormously.

Using the technique of lactonisation and with chromatographic sample analysis we have also been able to separate and therefore identify isomers. This is a huge advantage over techniques such as FAB/MS where the mode of sample ionisation precludes separation. Although this problem may be overcome with LC-ESI-MS/MS, as discussed in Chapter 6, some of the time saving advances in sample throughput would have to be sacrificed to ensure chromatographic separation prior to analysis.

Urine has provided an excellent medium for the analysis of acylcarnitines. It has been demonstrated here that samples are stable for long periods of time (2 years at 2°C) and provides a snapshot of the metabolic profile of an individual at the time of sampling. The

extension of this method to the analysis of dicarboxylic acylcarnitines (Chapter 4) and the analysis of acylcarnitine from blood spots (Chapter 5) will add greatly to the scope of these analyses and the ease of obtaining samples from neonates.

3.5 EXPERIMENTAL.

3.5.1 MATERIALS.

The authentic acylcarnitines used to compile Table 3.1, standard valproylcarnitine and 4-phenylbutanoylcarnitine (internal standard) were synthesised as reported earlier [1, 45] or, where commercial reagents were available, obtained from Sigma (St. Louis, USA). *N,N*-diisopropylethylamine was purchased from Aldrich (Gillingham, UK), ethyl acetate (distol grade) and acetonitrile (distol grade) from Fisons (Loughborough, UK), Analar water from BDH Merck (Poole, UK). Reacti-vials (1 ml) from Pierce (Chester, UK), analytical grade ion-exchange resins from Bio-Rad (Hemel Hempstead, UK) and isobutane (99.995% pure) from gas products (Finchamstead, UK).

3.5.2 EXTRACTION OF ACYLCARNITINES FROM URINE SAMPLES (ION EXCHANGE METHOD)

The method for the extraction of acylcarnitines from urine was based on the work of Norwood et al (1988) [46, 47]. A 1 cm diameter column was packed with formate ion-exchange resin (2 cm³), 100 - 200 mesh (Bio-Rad AG1-X8). This was converted to chloride form through elution of 1 M HCl (10 ml) and the column was equilibrated with distilled water. The urine sample (0.5 ml) was added to the head of the column.

Acylcarnitines and neutral and cationic material were eluted with distilled water (2 ml) and the eluant acidified with 100 ml of 1 M HCl.

Hydrogen form cation-exchange resin was then used to further purify the eluant from above. The cation-exchange resin, 100 - 200 mesh, Bio-Rad AG50W-8X (2 cm³) was packed into a 1 cm diameter column and the eluant was applied to the column.

HCl (0.01 M, 5 ml) and distilled water (5 ml) were used to elute neutral and loosely bound cationic species. The acylcarnitines were then eluted using 2 M NH_4OH in 20% aqueous ethanol. The first millilitre was discarded and the remaining eluant collected and freeze-dried. The resulting residue was dissolved in 0.5 ml of distilled water, transferred to a Reacti-vial and freeze-dried. The standard cyclization procedure was then carried out to produce acylcarnitine lactones for GC analysis [1].

3.5.3 STANDARD CYCLIZATION PROCEDURE.

Phenylbutanoylcarnitine (200 μl , 500 mg/l solution), the internal standard, was added to the extracted residue containing acylcarnitines and this was then dried under a stream of nitrogen. Acetonitrile (400 μl) and 80 μl DPA solution (*N,N*-diisopropylethylamine, 25 mg/ml) were added to the residue in a Pierce Reacti-vial and the vial was shaken for about 30 seconds. The mixture was then heated at 125°C for 35 minutes. The sample was then cooled to room temperature and dried under a stream of nitrogen. The resultant residue was taken up in 200 μl of ethyl acetate and filtered using a 2 μm filter and a glass syringe. The solution was stored in the freezer until analysed. A schematic of acylcarnitine cyclization is shown in Figure 3.1, which can be found opposite page 67.

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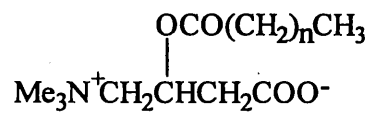
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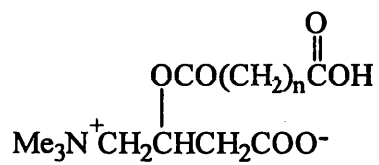
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CHAPTER 4.

ANALYSIS OF DICARBOXYLIC ACYLCARNITINES.



Monocarboxylic acylcarnitines



Dicarboxylic acylcarnitines

Figure 4.1 A structural comparison of monocarboxylic and dicarboxylic acylcarnitines both of which can be used in the diagnosis of neonatal metabolic disorders.

4.1 INTRODUCTION.

It has been possible to characterise a number of inherited metabolic diseases due to the presence of acylcarnitines derived from monocarboxylic acids at elevated levels in biological fluids and tissues of neonatal patients. In addition to the excretion of monocarboxylic acylcarnitines many disorders can be diagnosed by the identification of dicarboxylic acylcarnitines [1, 2, 3]. A structural comparison of monocarboxylic and dicarboxylic acylcarnitines is shown opposite in Figure 4.1. Disorders, which can be recognised from the dicarboxylic acylcarnitine profile from biological fluids, include the metabolic state of diabetic ketosis [4], riboflavin deficiency [5, 6] and prolonged fasting [7] and disorders of β -oxidation. This latter group of diseases will be addressed here. The production of dicarboxylic acylcarnitines in fatty oxidation defects is outlined in Figure 4.2 overleaf.

Dicarboxylic acylcarnitines have been analysed using a number of analytical techniques including high-performance liquid chromatography (HPLC) [8, 9]. In most HPLC methods acyl-CoA esters formed from enzymatic conversion of acylcarnitines are analysed [10] or the chromatographic properties of the acylcarnitines are enhanced through formation of their 4-nitrophenyl derivatives [11] (as described Chapter 1). Gas chromatography (GC) [12] and gas chromatography mass spectrometry (GC/MS) [13] have also been used for this analysis although the detected compounds are usually the acids released on hydrolysis of acylcarnitines. Direct analysis has been carried out using fast atom bombardment mass spectrometry (FAB/MS) [11, 14, 15], liquid chromatography mass spectrometry (LC-MS) [16], paper and thin layer chromatography, nuclear magnetic resonance (NMR) spectrometry [17] and a number of enzymatic and radioenzymic techniques (see Chapter 1).

The excretion of dicarboxylic acylcarnitines has been highlighted due to a significant increase in adipic- (C_6), suberic- (C_8) and sebacic- (C_{10}) dicarboxylic acid levels under a variety of conditions. It was demonstrated that medium-chain dicarboxylic acids are

LONG-CHAIN ACYL-CoA

LONG-CHAIN DICARBOXYLIC ACIDS (DCA)

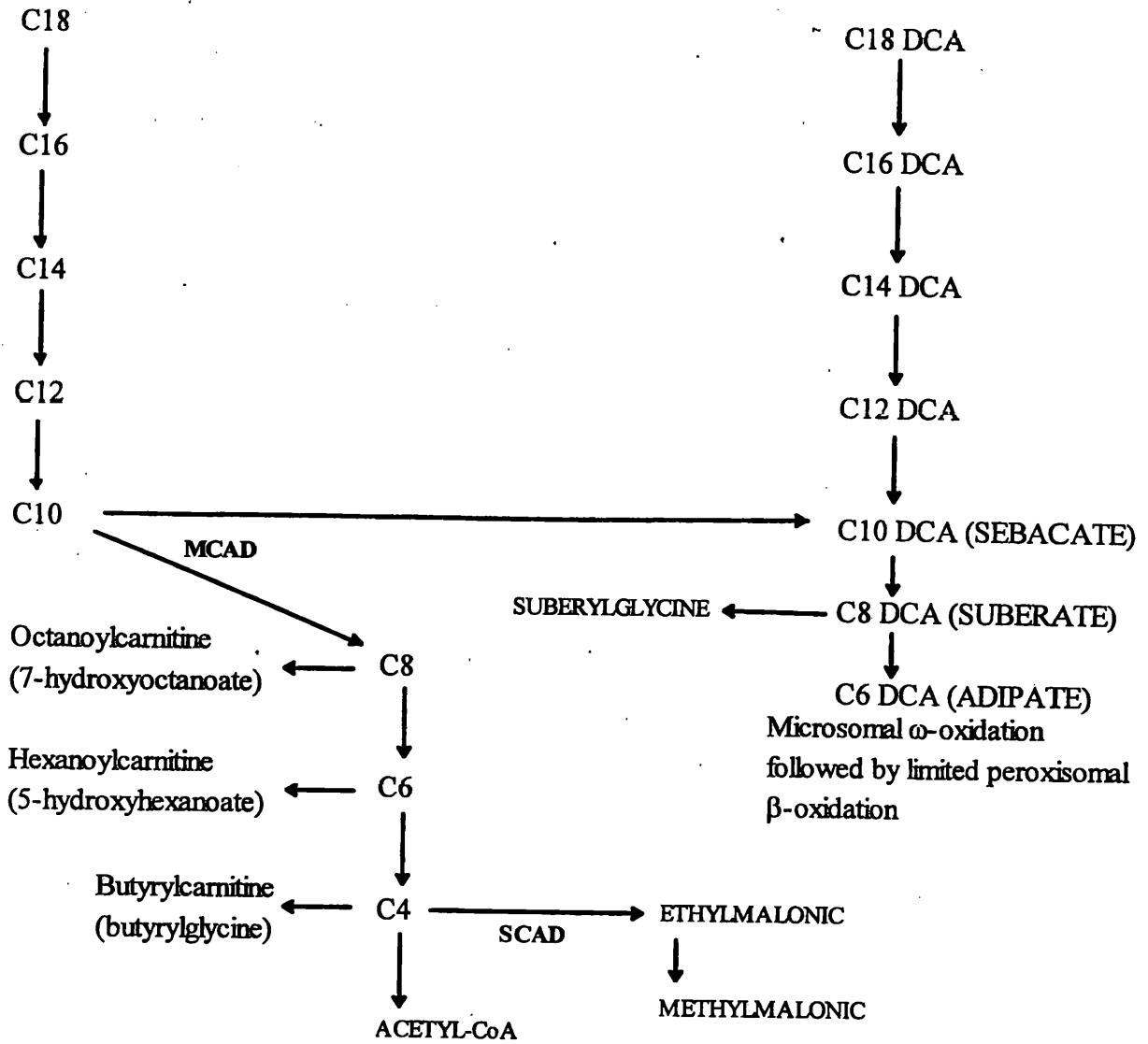


Figure 4.2 A schematic illustrating the production of dicarboxylic acids (DCA) and carnitine and glycine esters in fatty acid oxidation defects.

formed by cytochrome p450 mediated ω -oxidation of medium-chain monocarboxylic acids in the cytosol of the cell. Higher levels of activity were associated with C₁₀ and C₁₂ monocarboxylic acids than with C₆ and C₈ acids [18]. Patients with metabolic disorders, associated dicarboxylic acid excretion generally show a higher level of adipic (C₆) secretion than the longer chain lengths which would be predicted [19]. It has therefore been widely assumed [18, 19] that ω -oxidation is responsible for the formation of C₁₀ and C₁₂ dicarboxylic acids at elevated levels. These acids are then chain-shortened by β -oxidation to produce the C₆ and C₈ dicarboxylic acid profiles observed. Microsomal ω - and ω_1 -oxidation under normal conditions are responsible for 4-5% of fatty acid metabolism. In an earlier study, Kølvråa (1979) [20] demonstrated that in rats the dicarboxylic acids, C₁₄-C₁₈, from homogenated liver, when incubated, were even-numbered lower-chain length dicarboxylic acids. It has been shown that both purified mitochondria and peroxisomes use identical systems for the transport, activation and β -oxidation of mono- and dicarboxylic acids.

Table 4.1 Dicarboxylic acids that are characteristic of metabolic defects [19].

DEFECT	URINARY ORGANIC ACIDS
LCADD	Adipic, suberic, sebacic and C ₁₂ -,C ₁₄ - dicarboxylic acids
MCADD	Adipic, suberic, sebacic (saturated and unsaturated). Hexanoyl-, suberyl-, 3-phenylpropanoylglycine, octanoylcarnitine, 5-hydroxyhexanoic and 7-hydroxyoctanoic acid.
SCADD	Ethylmalonic, methylsuccinic, butyrylglycine, butyrylcarnitine.
LCHAD	Adipic, suberic, sebacic (saturated and unsaturated), 3-hydroxy C ₈ , C ₁₀ , C ₁₂ , C ₁₄ dicarboxylic acids.
MADD, GA II	Same as SCAD, MCAD, LCAD plus glutaric, 2-hydroxyglutaric, isovalerylglycine, isobutyrylglycine, isovalerylcarnitine, glutarylcarnitine.

Excretion of dicarboxylic acids is indicative of a number of metabolic disorders (Table 4.1) [19] and can therefore be utilized as a tool for the identification of disease (as with the mono-carboxylic acids). Since the same transport system is used as that studied for monocarboxylic acids, one can assume that dicarboxylic acids are present in the urine of patients with these disorders, and this has been demonstrated by a number of groups [14]. We aim to extend the analysis already available for simple acylcarnitines to dicarboxylic acylcarnitines. This would be a major advance, allowing some ill-defined dicarboxylic

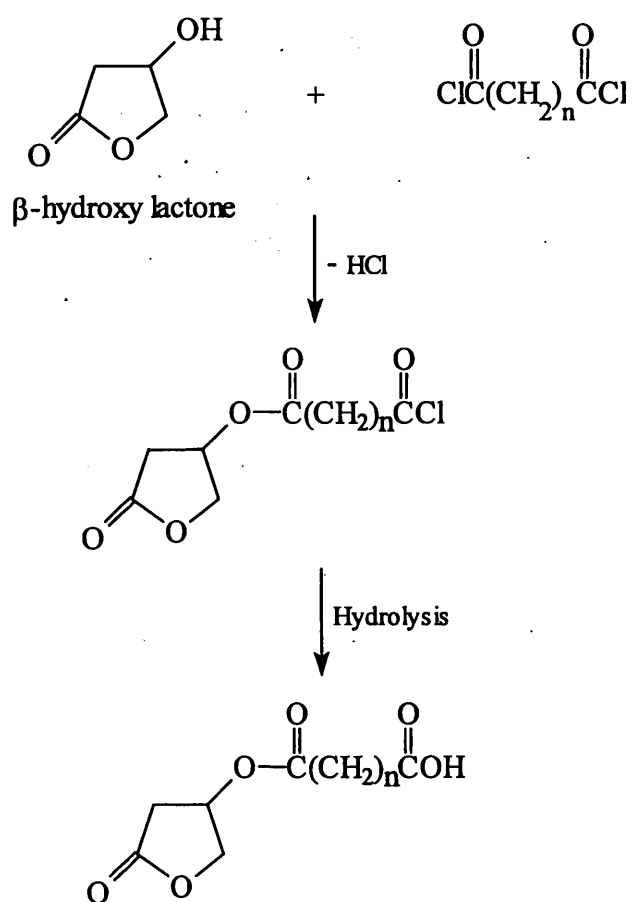


Figure 4.3 Synthesis of standard dicarboxylic acylcarnitine lactones. These standard dicarboxylic acylcarnitine lactones were used to gain spectrophotometric and chromatographic information as to the characteristics of these compounds.

acidurias to be investigated. A moderate generalized dicarboxylic aciduria (DCA) has been shown in 25% of 700 SIDS, siblings and controls [21]. The nature of the dicarboxylic acylcarnitines is likely to reflect the DCA, so that any progress towards a simple analytical procedure in this area will allow these unclear findings to be readily investigated.

4.2 RESULTS AND DISCUSSION.

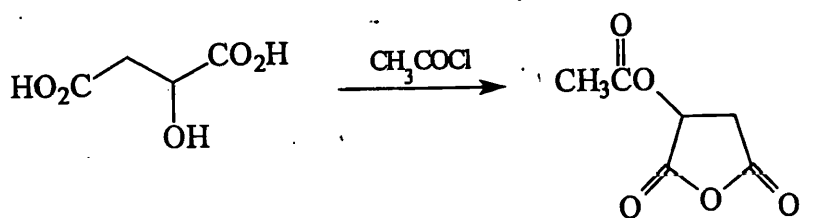
The aim of the experiments discussed here was to obtain a lactone derivative of a dicarboxylic acylcarnitine, from a clinical urine sample, that was amenable to analysis by GC and GC/MS. The primary stage towards achieving this goal was the synthesis of a model acylcarnitine lactone from which chromatographic and mass spectral information could be obtained. With such data, successful later extraction and derivatization of dicarboxylic acylcarnitines could be assessed and confirmed.

The formation of these standard compounds involved the synthesis of a lactone ring, β -hydroxylactone, in the first instance and its reaction with the relevant diacid dichlorides to produce the required authentic compounds (Figure 4.3).

The synthesis of the β -hydroxylactone (Section. 4.3.2) was a key element of this part of the work [22, 23]. A scheme of this synthesis is shown as Figure 4.4, overleaf. The standard dicarboxylic lactones were required to obtain further information as to the expected products of the extraction and derivatization of dicarboxylic acylcarnitines from biological fluids. In this way diagnostic structural information via the spectrometric techniques of IR and NMR and that available from GC and GC/MS would be available.

The initial step in this synthesis was the formation of the cyclic anhydride (Figure 4.4 (a)). This was formed as described with a high yield (91%) and its presence confirmed through IR and NMR spectroscopy as well as its melting point. All analytical data were compared to those recorded by Lowes [23] for confirmation. The next stage of the synthesis involved

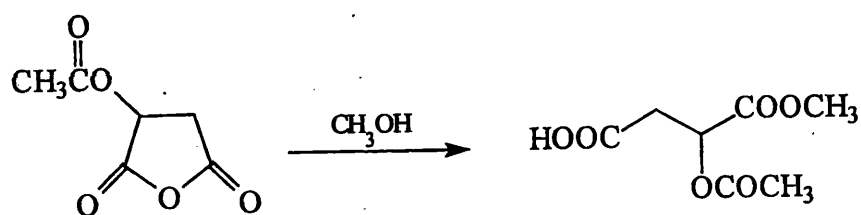
a



dl-malic acid

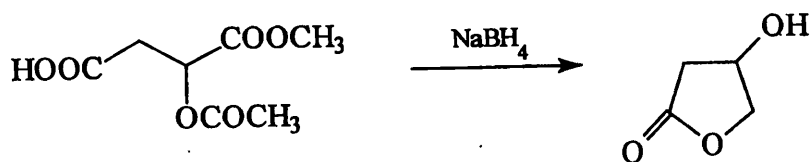
cyclic anhydride

b



diester-acid compound

c

 β -hydroxy lactone

4c

Figure 4.4 Schematic illustrating the synthesis of the β -hydroxy lactone. The experimental procedure involved in this synthesis is outlined in Experiment 4.3.2 (1 - 3)

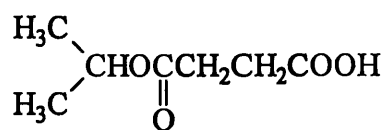
the production of a diester acid compound (Figure 4.4 (b)). This synthesis was also successful, with a satisfactory yield of 65%, and was validated in the same manner as the cyclic anhydride. The final stage of the reaction was the reduction of the diester-acid compound to the required hydroxy lactone (Figure 4.4 (c)). This stage of the synthesis presented a number of problems.

It was found that this reaction required scrupulously dry conditions to be successful. In particular if the ^tBuOH solvent was wet, the reducing agent was ineffective and starting materials were recovered. The detrimental effect of water was minimised by drying and redistillation of the tertiary butanol (b.p. 82°C), taking care to discard the first distillate which results from an azeotropic mixture with water (b.p. 79°C). The distillate was stored for not more than 24 hours, over molecular sieve. With the measures described here, synthesis of the hydroxy lactone was achieved. Though the yield of the final product was low (25%) it was similar to that reported previously [24] and the final product was shown analytically to require no further purification.

Due to the problems associated with the final stage synthesis of the hydroxylactone and its poor yield, it was decided to develop a method for the subsequent reaction with a diacid dichloride on a more readily available alcohol, in place of the hydroxylactone. In this way any necessary modifications to the procedure, for the synthesis of model acylcarnitine lactones could be made and assessed without expending the hydroxylactone.

The alcohols chosen were propan-2-ol and cyclopentanol. The former was selected because it is a simple, secondary alcohol and the latter chosen because it is secondary and cyclic, in common with the hydroxylactone. Both alcohols were used in the reaction as described in Experiment 4.3.3 with the required products illustrated in Figure 4.5 overleaf. Analysis was carried out on a VG 20-250 quadrupole instrument with a direct insertion probe in the electron ionization (EI) mode. A response at m/z 161 in the case of propan-2-ol was assigned to the $[M + H]^+$ of the required product (its actual relative molecular mass is 160). The protonated molecule can be explained by self-chemical ionization of the

(a)



(b)

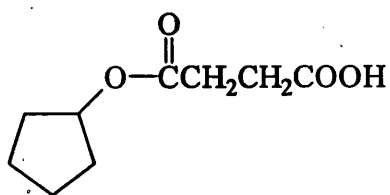


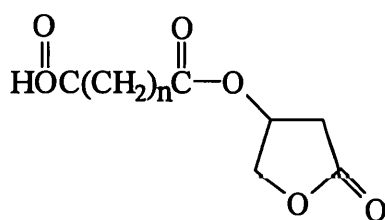
Figure 4.5 The required products from the reaction of succinyl dichloride with either (a) isopropanol or (b) cyclopentanol.

These compounds when synthesised were used to develop a method for the later synthesis of model dicarboxylic acylcarnitine lactones

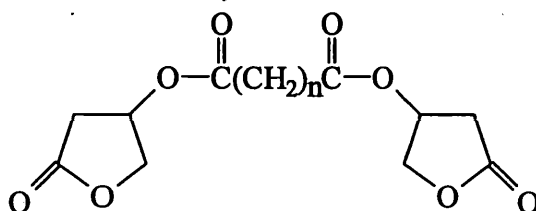
sample as can occur when too much sample is present on the probe. A peak at m/z 203 can also be clearly seen above that of the noise level. This may be assigned to the $[M + H]^+$ ion of a by-product in the form of the diester of the required product. In the reaction utilizing cyclopentanol similar results were obtained. The mass spectrum obtained again demonstrated evidence of self-chemical ionization (m/z 187) of the product and also self-chemical ionization (m/z 255) of the equivalent diester, at a probe temperature of 100°C.

On completion of these test reactions it was predicted that three products would result from the analogous reactions of a diacid dichloride with the hydroxylactone. These are depicted overleaf in Figure 4.6. The ratios of each would depend on the reaction conditions utilized. They were the required lactone (4.6.a) and two contaminants: the diester (4.6.b) mentioned above and the parent diacid (4.6.c), because the diacid derived from the diacid dichloride was present in excess in the reaction mixture. Minimization of the diester was achieved through the order of addition of reactants in the test reactions. Addition of the alcohol to the diacid dichloride ensured that at no stage in the synthesis was there an excess of alcohol present thus minimizing the potential for attack at both ends of the diacid dichloride. For the same reason the molar ratio of acid chloride groups to alcohol was also greater than unity resulting in a high level of the diacid in the product after work-up. The presence of the diacid was confirmed by Thin Layer Chromatography and NMR spectroscopy. In the case of TLC a smeared spot of material over a wide R_f range characterised the presence of diacid over a wide R_f range. In the case of the NMR spectroscopy the presence of extra carbonyl group carbon atoms in the ^{13}C NMR was indicative of this excess.

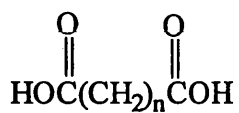
A base wash of the product mixture was introduced in order to remove this diacid contamination. In this case the diacid remains in the aqueous layer as a carboxylate ion while the required lactone, being less hydrophilic was extracted into the organic, dichloromethane layer. The reaction was then applied to the synthesis of a number of standard dicarboxylic acyloxylactones with reaction conditions that were identical to those of the model compounds. Infrared analysis was carried out in the 4000-600 cm^{-1} range



(a) Required product



(b) Unwanted diester compound



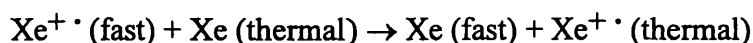
(c) Diacid (from excess of acid chloride)

Figure 4.6 Three potential products from the synthesis of dicarboxylic acylcarnitine lactones. These were determined from experiments into the reaction of diacid dichloride with the model alcohols illustrated in Figure 4.5.

and the spectrum obtained proved very diagnostic (as detailed in the figure legend to Figure 4.7) of the test reaction products and of the synthesised acyloxylactones. It was also possible to monitor the hydrolysis of the side-chain acid chloride to the carboxylate group by this method (Figure 4.7).

Analysis of the standard acyloxylactones was also carried out using the technique of Fast Atom Bombardment (FAB). In this technique a small amount of the analyte is placed on the metal coated tip of a probe and dissolved in a drop of a matrix solvent that is polar and fairly involatile (e.g. glycerol). The probe is then inserted in a low pressure source. A beam of atoms is produced through the ionization of a gas, usually xenon, argon or helium. These ions are accelerated through an electrical field and the resulting fast ions are focused through a gas chamber where the process of charge exchange occurs to yield fast atoms [24]. This process is illustrated, below.

The production of Fast Atoms in FAB



These fast atoms are then used to bombard the probe tip and hence the sample. The large kinetic energy of the atoms is used to ionize or volatilize the sample. Through variation of the electric gradient over the probe it is possible to select positive or negative ions for analysis. FAB has been shown as a valuable method for the analysis of polar involatile, and thermally unstable compounds. The lactones prepared are stable but they were considered sufficiently polar to provide FAB mass spectra.

The FAB spectrum obtained from the bombardment of suberyl lactone in a glycerol matrix resulted in detection a protonated molecule $[\text{M} + \text{H}]^+$, at m/z 259. Glycerol fragments and adducts can also be seen in this spectrum as can glycerol/sample or glycerol/fragment adduct ions. In the ^1H NMR spectrum of succinyl lactone (as Figure 4.8, page 97). Both solvent and diacid contamination are present and the required product is also detected. These contaminants are annotated in the spectrum of Figure 4.8 and the ppm assignments for

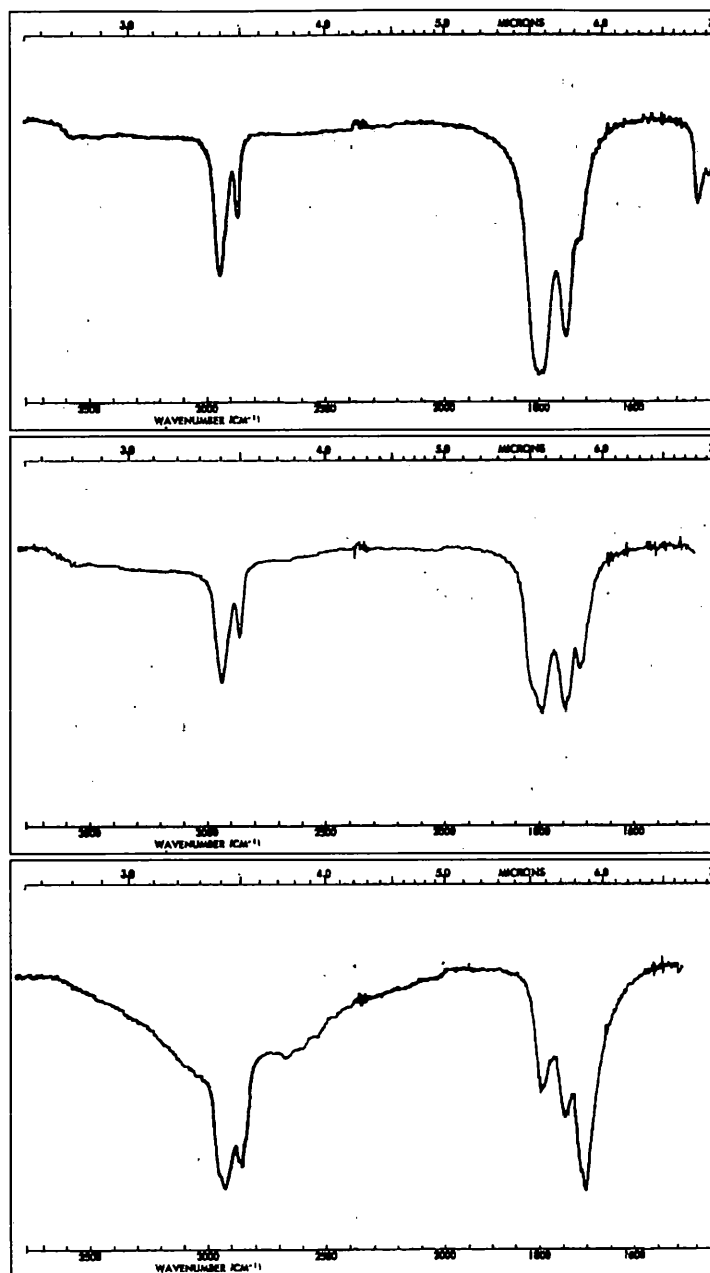


Figure 4.7

- (a) Initial IR with very little broadening in the acid ($3500\text{--}2500\text{ cm}^{-1}$) stretch region. There is a strong chloride (C=O , 1800 cm^{-1}) carbonyl peak present and the ester carbonyl (1740 cm^{-1}) is also visible.
- (b) Slight hydrolysis has occurred showing a broadening of the acid stretch region, the chloride peak has diminished and an acid (1700 cm^{-1}) carbonyl peak beginning to show and the ester peak more clearly defined.
- (c) Hydrolysed product. A lactone (1790 cm^{-1}) carbonyl has now become visible in the region where the chloride had absorbed, and the chloride peak has gone completely. The ester is not much changed with respect to (b) and the acid carbonyl is now the dominant peak in the region. The broadening of the $3700\text{--}2300\text{ cm}^{-1}$ acid OH stretch region confirms the presence of carboxylic acid.

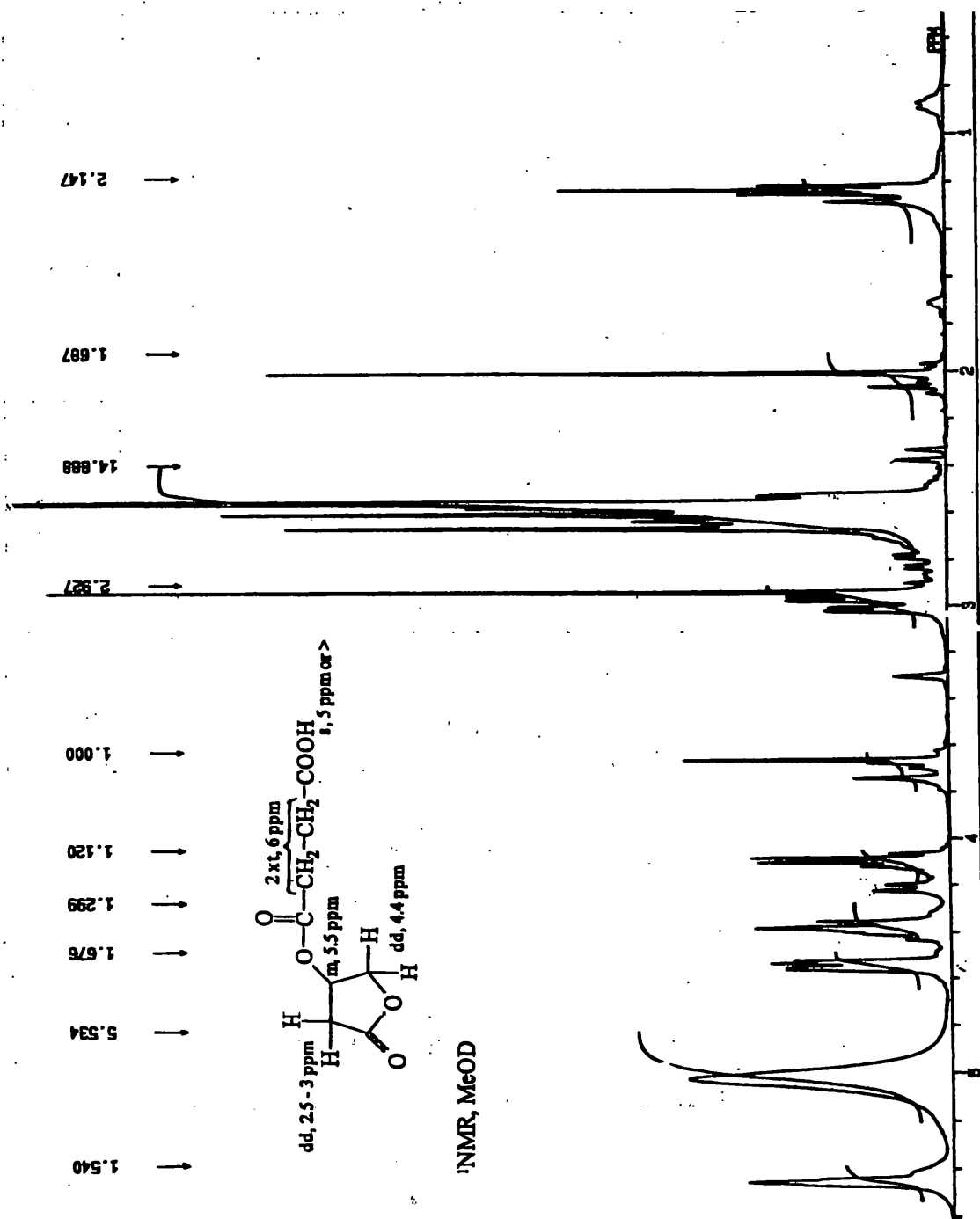


Figure 4.8 ¹H NMR succinylcarnitine lactone

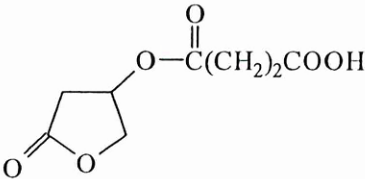
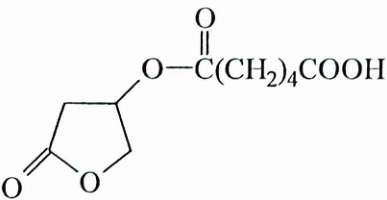
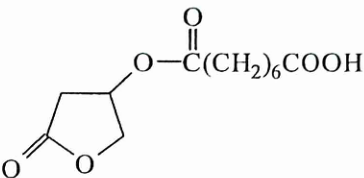
Compound name	Structure	Retention time
C4 Succinyl lactone		11.45 min
C6 Adipyl lactone		14.56 min
C8 Suberyl lactone		17.21 min

Figure 4.9 Retention times (GC, BP5 column), and structural information for the three dicarboxylic acylcarnitine lactones (as TMS derivatives)

succinylcarnitine lactone are also annotated and illustrated. On completion of the base wash, analysis of the product was performed by GC and GC/MS with successful identification of the required product.

If one considers the structure of the compound for GC analysis, the effect of the free acid capping the hydrocarbon side-chain will have a detrimental effect on the quality of the chromatography obtained. This is due to polar interactions with the stationary phase in the silica capillary which causes zonal spreading over a wider area and the compound is detected as a broad, tailing peak. To overcome this problem of poor peak shape a derivatization was performed on the molecule by means of trimethylsilylation (TMS) of the terminal free acid. This derivatization, using BSTFA, improved the peak shape significantly.

In the same way chromatographic and mass spectral information was generated by GC/EIMS and GC for the TMS derivatives of succinyl- (C_4), adipyl- (C_6) and suberyl- (C_8) lactones synthesised via the β -hydroxy-lactone. The structures and retention times for these compounds are illustrated in Figure 4.9, opposite.

With these data generated the next step was to synthesise standard dicarboxylic carnitines (Experiment 4.3.5) which could then be cyclised via the standard lactone preparation. The synthesis was carried out using adipyl dichloride as likely to be typical of the range of compounds of interest and to react in a manner, which could be considered normal for this group. The synthesis (Figure 4.10, overleaf) produced the required acylcarnitine but with very low yields though analysis by FAB showed an $[M + H]^+$ ion was present at m/z 290. NMR (^{13}C) spectroscopy was also used to confirm the formation of the acylcarnitine. The carbonyl ($C=O$) region in a ^{13}C spectrum is approximately 160-200 ppm and in the case of analysis of the required adipylcarnitine one would expect to observe three peaks. The first corresponds to the terminal carboxyl group on the carnitine backbone of the molecule, the

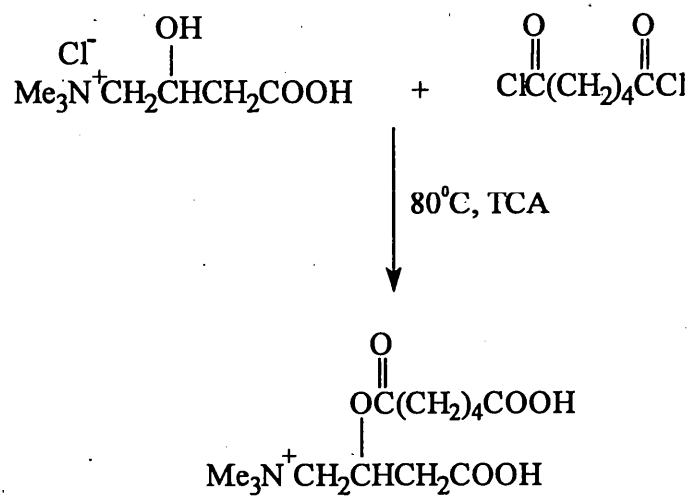


Figure 4.10 The synthesis of adipylcarnitine via the diacid dichloride
(Experiment 4.3.6)

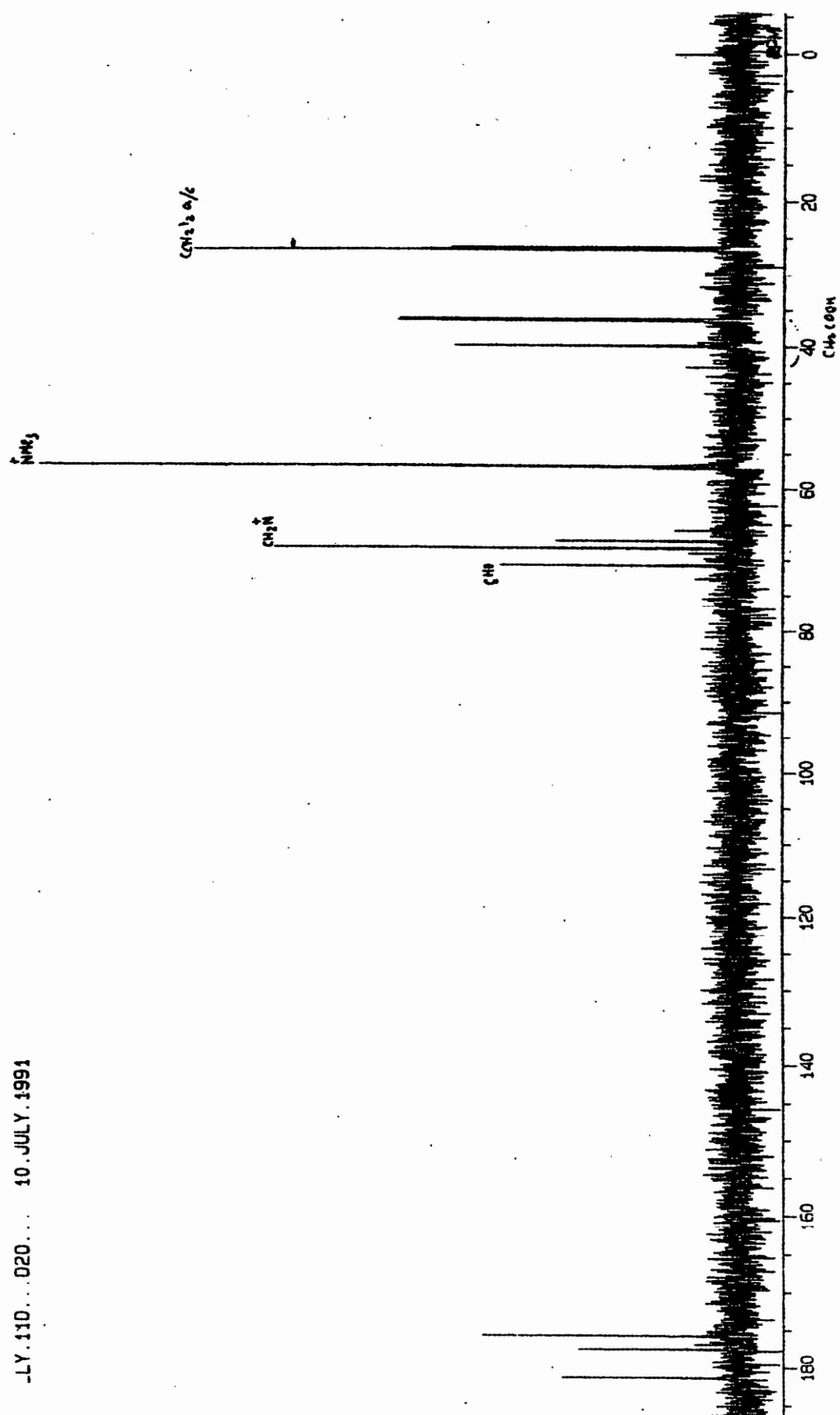
second to the side-chain free acid and the third to the side-chain ester carbonyl group. In the unpurified material there were at least four peaks identified in the carbonyl region. On purification comprising of a reprecipitation from propan-2-ol these extra peaks were no longer detected and three carbonyl peaks can be clearly defined in this area (Figure 4.11, page 100).

It was noted during work on the lactone reaction that a large tailing peak was seen in the area of 7.5 min (GC) which on TMS derivatization sharpened its shape and proved to have half the area of the internal standard peak. GC/MS analysis showed that adipic acid was not the source of this peak. Phenylbutanoic acid was then tested in a similar manner and this proved to be the unknown peak. On analysis of the original phenylbutanoylcarnitine (synthesised 1988) it was found to be contaminated with a large amount of its parent acid which indicated the breakdown of the phenylbutanoylcarnitine over approximately three years.

Attempts to cyclize the synthesised adipylcarnitine proved unsuccessful. It had been shown in the work of Lowes (unpublished) that the success of the lactonization was sensitive to the purity of the acylcarnitine and its concentration, with higher concentrations producing lower cyclization yields than smaller concentrations. It was suggested that, at high concentrations, intermolecular reactions leading to polymerization might predominate over the required intramolecular reaction. It was considered that the purity of the acylcarnitine could also be a factor in the results observed here and therefore an alternative synthesis pathway for the dicarboxylic acylcarnitine, which has been reported at high yield and reasonable purity, was proposed. Experiments (Experiment 4.3.6) were carried out in order to produce a dicarboxylic acylcarnitine (adipylcarnitine) in a purer form and in larger amounts than had previously been achieved, as shown in Figure 4.12, opposite page 101.

This purer sample of adipylcarnitine was subjected to the cyclization procedure followed by production of the TMS derivative. This sample was analysed after overnight storage in the freezer. The resulting mass chromatogram consisted of one peak with a retention time of 8.3 minutes, identified as the TMS derivative of adipic acid. This identification was

Figure 4.11 The ^{13}C NMR spectrum used to confirm the synthesis of Adipylacrnitine



made on the basis of the characteristic loss of 15 mass units from the molecular ion, m/z 275, and also using a library search. Analysis using GC alone gave rise to a chromatogram with two peaks. The peak corresponding to adipic acid and a later eluting peak with a retention time of approximately 14 minutes. The GC/MS analysis was carried out with a number of samples of the adipylcarnitine with a response detected at a retention time of approximately 15 minutes. This corresponds to the retention time window for adipylcarnitine lactone as detailed in Figure 4.9, opposite page 98. This was a very low response identified by producing mass chromatograms of predicted fragments.

The above experiment was later repeated with one change, that is, analysis of the TMS derivative was carried out immediately rather than after the sample had been stored in the freezer, with substantially different results. There was only one major peak in the resultant chromatogram, rather than that of the diacid TMS, and this was detected in the 15 minute region where the adipylcarnitine lactone would be expected. There was also a peak in the region where the diacid TMS was expected but this was very small compared to the peak described. After airtight storage for 24 hours in the freezer, GC analysis showed that the peak of interest, 14.7 minutes had approximately halved in size. Accompanying this breakdown, a peak had appeared at 8.5 min, which proved to be the diacid TMS; it was also noted that there was a peak at a retention time of 17.2 min, which had not been present in the initial chromatogram.

Analysis by GC/MS was not possible at this stage and when the sample was analysed, by GC 48 hours later, it was found that the peak at 17.2 min had disappeared almost entirely. However, the peak for the diacid and the peak at 14.7 min had halved size in intensity and a number of smaller peaks had appeared. At this point the sample was not considered viable for GC/MS. It was proposed that the large peak may have been due to the required compound but some decomposition of the product had obviously occurred in the solution containing excess derivatizing agent.

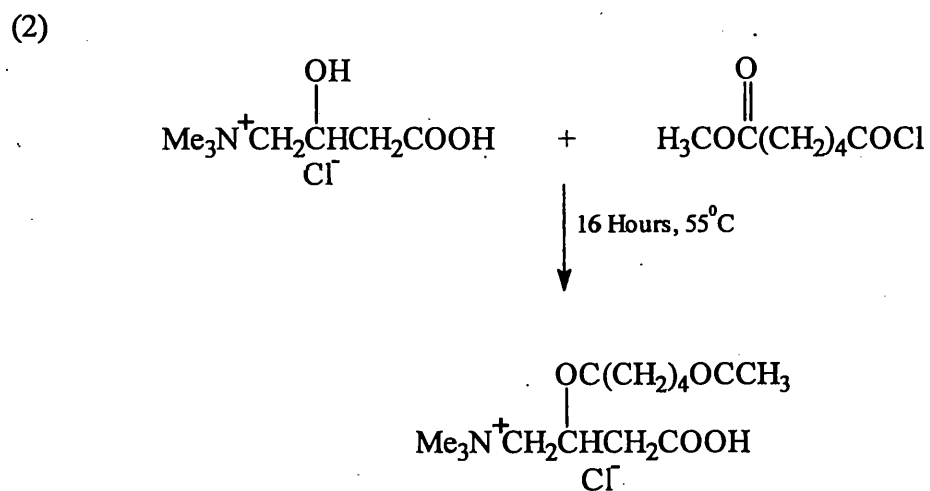
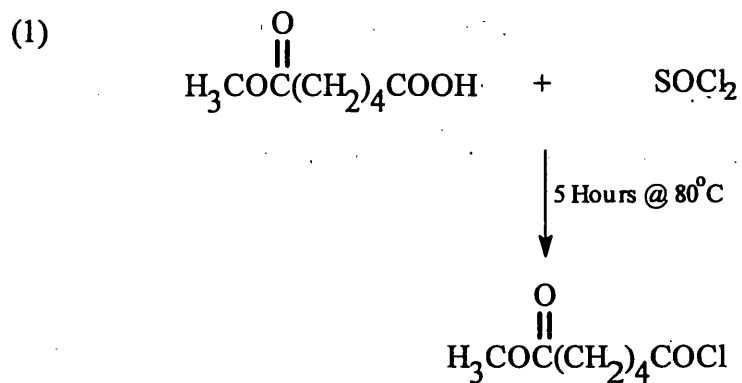
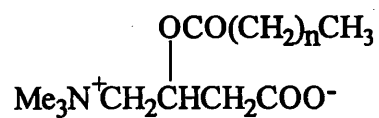


Figure 4.12 The synthesis of adipylcarnitine via the monoester monochloride (Experiment 4.3.7)

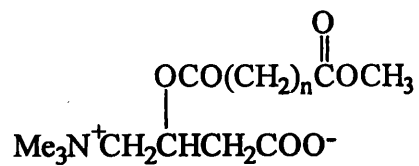
From these experiments it was clear that a problem existed either in the cyclization stage of this procedure or in the stability of the lactones formed. Where analytical data have suggested that the required acylcarnitine is present and that the sample has little other contamination, GC and GC/MS studies have failed to give definitive identification of lactones from the cyclization. This led to the possibility that the nature of the molecule itself was in some way preventing the cyclization from occurring efficiently, if at all. In a comparison of the structures of mono- and dicarboxylic acylcarnitines the obvious difference was the presence of an extra acid group in the side-chain of the latter. To ascertain the effect of the free carboxylic acid group, the synthesis of a model intermediate molecule was proposed (Figure 4.13, overleaf).

The model molecule, instead of having a free terminal acid group, has a methyl ester group but maintains the extra bulk of the dicarboxylic acid side chain. Thus with the terminal acid capped in this way it would be possible to assess the role of the free acid group in hindering the cyclization, if any. The compound chosen for synthesis was adipylcarnitine mono-methyl ester as this was the equivalent intermediate to the adipylcarnitine (dicarboxylic acylcarnitine) on which most of the previous work has been carried out.

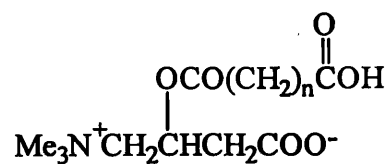
Three methods, FAB, NMR (both proton and carbon thirteen) and GC (after cyclization) were used to obtain analytical information on the required product for this experiment. The relative molecular mass of the methyl ester is 303. In analysis by FAB this would appear in the FAB^+ spectrum as an $[\text{M} + \text{H}]^+$ ion at m/z 304. The peak at m/z 304 is large in this spectrum which reflects to some extent the purity of the product. The main peaks in the mass spectrum are those at the m/z values of 58, 85, 100, 111, 144, and 162; possible structures for these m/z values are indicated below. The base peak in this spectrum occurs at m/z 162 which corresponds to the $[\text{M} + \text{H}]^+$ of carnitine (possibly derived from some unreacted carnitine hydrochloride) and to a potential fragment ion. The peaks at m/z 85 and 144 are usually present in the electron impact mass spectra of acylcarnitine lactones representing fragmentations to yield the lactone ring (m/z 85) and a McLafferty rearrangement of the ring and the beginning of the side chain to give m/z 144. Peaks at



Monocarboxylic acylcarnitines



Dicarboxylic acylcarnitine mono-methyl ester



Dicarboxylic acylcarnitines

Figure 4.13 Three structures for comparison, (a) Monocarboxylic acylcarnitine, (b) The model methyl ester compound, (c) Dicarboxylic acylcarnitine

m/z 85 and 144 in the FAB spectra of acylcarnitines themselves are proposed to have different structures as shown in Figure 4.14, overleaf. The other fragments are equivalent to those seen in the spectra of authentic acylcarnitines, hence confirming the identity of the prepared substance.

The ^1H NMR spectrum of the methyl ester contained the expected resonances from the required product though there were extra peaks present which, may be assigned to unreacted carnitine. It was possible from the integrals to estimate the approximate purity of the methyl ester (60%). This assignment was aided by the ^{13}C NMR spectrum, which, also indicated a lack of by-product contamination. The ^{13}C NMR spectrum of the product clearly demonstrated the presence of three carbonyl groups, though these have not been individually assigned. The carbonyl groups were in the region of 165 - 175 ppm.

It was proposed to study the effect the presence of free acid would have on the cyclization of the methyl ester (intermediate compound) (Experiment 4.3.8.1, samples prepared as in Table 4.2), as mimicking that capping the end of the dicarboxylic side chain. To do this the cyclization step was carried out in the presence of an ammonium acetate solution. Approximately equimolar solutions of each reagent was used, in the case of the acetate this was carried out to mimic the molar quantities of the terminal free acid usually present. All the samples were initially analysed by GC and the resultant chromatograms were used to monitor the efficiency of the cyclization of the methyl ester relative to that of the added octanoylcarnitine. Octanoylcarnitine may be considered the internal standard in these experiments as its cyclization has been more extensively studied.

Following GC and GC/MS analysis of the resulting solutions the lactone of the synthesised methyl ester was found to have a retention time of 13.3 min. It was however detected with a lower peak height than was expected from the previously studied monocarboxylic acids; this at least demonstrates that the derivatization has been successfully achieved. The other peak in the chromatogram (5.44 min) was, after GC analysis of the initial reactants in the synthesis of the methyl ester, shown to coincide with the retention time of adipyl

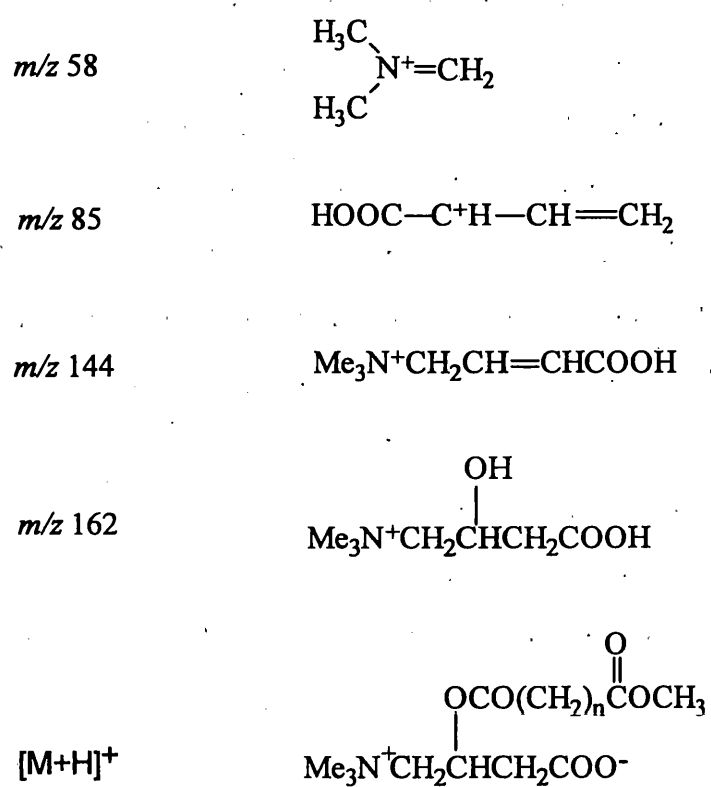


Figure 4.14 Key fragments from FAB⁺ analysis of the Methyl Ester intermediate (m/z 304)

monomethyl ester. The presence of this compound would indicate that the initial reaction did not go to completion and this would partly explain the lower peak height recorded. A second sample containing all three of the compounds being studied (the methyl ester, octanoylcarnitine, ammonium acetate) was subject to the usual cyclization conditions. In the resulting chromatogram the octanoylcarnitine lactone peak was of the expected peak height and shape. The ratio of the methyl ester to octanoylcarnitine lactones was shown (by peak height) to be approximately 1:6. This result could be explained through contamination or poor yield of the methyl ester, though as described earlier the ^1H and ^{13}C data suggest that the synthesized methyl ester was approximately 60% pure. Therefore this result suggests that the cyclization of the model methyl ester appears to be hindered. This low efficiency of cyclization of the methyl ester was also recorded in a third sample. It was also found that the presence or absence of ammonium acetate did not affect the efficiency of production of methyl ester lactone. The free acetate ion also had little or no effect on the cyclization of octanoylcarnitine alone.

Experiments replicated the results of the original samples in that the reagents (methyl ester: octanoylcarnitine), which are approximately equimolar in concentration, gave peak areas in a ratio of approximately 1:6.

To assess the effect of the free acid on already cyclized lactones, ammonium acetate was added after cyclization was complete. Its effect (if any) was then monitored to assess the stability of the lactones to free acid groups in solution. This demonstrated that ammonium acetate had no noticeable effect on the already cyclized octanoylcarnitine lactone, with analysis by GC.

In a study to assess the effect of acylcarnitine concentration on the efficiency of lactonization (Experiment 4.3.8.2, samples prepared as in Table 4.3), the conditions from experiments containing all three reagents from above were replicated, but using concentrations ten times lower. This was effected because the acylcarnitine concentrations were higher than those normally used in the cyclization reaction. From the most

concentrated sample, the chromatogram demonstrated the methyl ester to octanoyl carnitine ratio as 1:6 by peak area, as seen earlier. The peaks were detected at the retention times of 11.8 min (octanoylcarnitine lactone) and 13.94 min (the lactone of the methyl ester). The other samples with lesser concentrations in the study also demonstrated these ratio and retention times. This therefore suggests that the concentration of the reagents in a sample for cyclization (at the levels shown in these experiments) does not affect the efficiency of cyclization.

In summary, the analysis carried out in this experiment suggests that the dicarboxylic acylcarnitines would be expected to cyclize far less efficiently than their equivalent monocarboxylic acids, under the standard conditions employed. Though the free acid group may contribute in some way to their slow rate of cyclization external COO^- has no effect. The main problem seems to be the effect of an additional polar functional group in the acyl side-chain. This group may constrain the acylcarnitine to a configuration that cannot readily undergo the cyclization, i.e. unfavourable association may occur between the acid group in the side-chain and the ammonium group.

Given that the dicarboxylic acylcarnitines and monocarboxylic acylcarnitines are unlikely to be cyclize efficiently under the same set of conditions, this line of research was given a lower priority than the development of the standard procedure for analysing dried blood spots.

4.3 EXPERIMENTAL.

4.3.1 MATERIALS.

Malic acid, acetonitrile (distol grade) and ethyl acetate (distol grade) were obtained from Fisons (Loughborough, UK). Acetyl chloride, dioxane, sodium borohydride, thionyl chloride, toluene, *N,N*-diisopropylethylamine, adipyl mono-chloride and trichloroacetic acid were purchased from Aldrich (Gillingham, UK). Methanol and chloroform were acquired from BDH Merck (Poole, UK) also acetone (Analar), ammonium acetate, sodium carbonate and sodium hydrogencarbonate. The diacid dichlorides of succinic, adipic and suberic acids and the corresponding parent dicarboxylic acids, were obtained from Aldrich, as were the alcohols propan-2-ol, cyclopentanol, tertiary butanol and the derivatization agent BSTFA. Reacti-vials (1 ml) were purchased from Pierce (Chester, UK), acrodisc filters (0.2 μm) from Gelman (Northampton, UK) and glass syringes from Weber Scientific (Teddington, UK). Hydrochloric acid, dichloromethane and diethyl ether were obtained from Rhone-Poluenec (Manchester, UK). Phenylbutanoylcarnitine was synthesised via phenylbutanoic acid (Aldrich) and dl-carnitine hydrochloride (Aldrich) using previously reported methods [25, 26]. Octanoylcarnitine was purchased from Sigma (St. Louis, USA).

4.3.2. SYNTHESIS OF THE β -HYDROXY LACTONE

The synthesis was carried out in three steps. The preparation of a cyclic anhydride, its conversion to a diester acid compound and its reduction to the hydroxylactone were carried out by a modification to a literature method (Figure 4.4) [22].

4.3.2.1 CYCLIC ANHYDRIDE SYNTHESIS.

Malic acid (13.4g, ^1H NMR Appendix B1) was dissolved in redistilled acetyl chloride (120 ml) and stirred overnight at 50°C. The solution was then cooled, gravity filtered and rotary

evaporated. The resultant yellow solid was then washed with ethanol-free chloroform and dried to yield an off-white solid. Melting point 80-83°C, yield 13.2g, (91%).

Infrared data: 1700 cm^{-1} (ester), 1820 cm^{-1} , 1760 cm^{-1} (C=O, anhydride).

^1H NMR, CDCl_3 , 90MHz. δ 2.2 ppm (s, 3H, CH_3COO), 3.0 ppm (dd, 1H, H-2b), 3.4 ppm (dd, 1H, H-2b), 5.5 ppm (dd, 1H, H-3) - Appendix B1.

4.3.2.2 DIESTER-ACID COMPOUND SYNTHESIS.

The cyclic anhydride (12.46g, ^1H NMR Appendix B2) was dissolved in methanol (160 ml) and stirred at room temperature, overnight. The solution was then rotary evaporated, washed with toluene and dried to yield a white solid. Melting point 56-58°C, yield 11.03g, (62.5%).

IR: 3400 - 2900 cm^{-1} (OH stretching), 1700 cm^{-1} (acid, COOH), 1720 cm^{-1} (ester stretching, C=O), 1230 cm^{-1} (ester stretching, C-O).

^1H NMR, CDCl_3 , 90MHz. δ 2.1 ppm (s, 3H, CH_3COO), 2.9 ppm (d, 2H, CH_2), 3.7 ppm (s, 3H, CH_3OCO), 5.5 ppm (t, 1H, CH), 10.3 ppm (s, 1H, COOH).

4.3.2.3 HYDROXY-LACTONE SYNTHESIS.

Sodium borohydride (3.35g) was added to freshly distilled tertiary butanol ($^t\text{BuOH}$, 50ml) and this was then heated to reflux. The diester compound (4.2g) was dissolved in a mixture of $^t\text{BuOH}$:methanol (18:3.5 ml). This solution was then added to the sodium borohydride/ $^t\text{BuOH}$ and kept under reflux for 20 hours yielding a viscous liquid. The reaction was quenched by the addition of 7.8 ml of redistilled acetyl chloride in 110 ml of ethyl acetate and filtered. The filtrate was then neutralised with NaHCO_3 , filtered and then rotary evaporated which left a clear product which on freezing (for storage) formed crystals (1.12g, 25%).

IR: (Appendix B1) 3700-3000 cm⁻¹ (OH stretch), 3000-2900 cm⁻¹ (C-H stretch), 1780 cm⁻¹ (lactone, C=O stretch) – Appendix B3.

¹H NMR, 90 MHz, CDCl₃. δ 2.3-3 ppm (m, 2H, H-a/b), 3.9 ppm (d, 1H, OH), 4.2-4.5 ppm (m, 2H, H4 a/b), 4.7 ppm (m, H, H-3). The ¹³C spectrum of the β-hydroxy lactone is included as Appendix B4.

GC retention time for the synthesised hydroxy lactone was recorded at approximately 3 minutes. The GC conditions for this analysis were as follows: a cold-on-column was used with a temperature program of an initial temperature 87°C, which was ramped to 260°C at 10°C per minute. This upper temperature was then maintained for 5 minutes.

The GC, IR and NMR data suggested that the final product was reasonably pure.

4.3.3 SYNTHESIS OF MODEL ESTERS BY REACTION OF VARIOUS ALCOHOLS WITH DI (ACID CHLORIDES).

4.3.3.1 GENERAL PROCEDURE.

Diacid dichloride (2 mmols) was added to diethyl ether (30 ml) and the relevant alcohol (2 mmols) was added to this solution. The mixture was stirred at room temperature overnight and then refluxed for 2.5 hours. When cooled the mixture was rotary evaporated and hydrolysed by standing open to air overnight.

The experimental procedure given here was applied to the secondary alcohols, propan-2-ol and cyclopentanol, which were taken as models for the hydroxylactone.



4.3.3.2 ANALYSIS OF PRODUCTS.

(A) REACTION WITH PROPAN-2-OL.

IR: 2700-3500 cm^{-1} (COOH str), 3000-2900 cm^{-1} (C-H stretch), 1740 cm^{-1} (ester, C=O), 1790 cm^{-1} (chloride, C=O stretch) the latter due to incomplete hydrolysis of the product – Appendix B6.

GC/EIMS: m/z 161 (35%, $[\text{M} + \text{H}]^+$), m/z 143 (22%, $[\text{M} + \text{H}]^+ - \text{H}_2\text{O}$), m/z 119 (50%, $[\text{COOH}(\text{CH}_2)_2\text{COOH}_2]^+$), m/z 101 (100%, m/z 119 - H_2O), m/z 203 ($[\text{M} + \text{H}]^+$ diester) – Appendix B5.

(B) REACTION WITH CYCLOPENTANOL.

IR: 2500-3500 cm^{-1} (COOH str), 3000-2900 cm^{-1} (C-H stretch), 1740 cm^{-1} (ester, C=O), 1790 cm^{-1} (chloride , C=O stretch). The latter due to incomplete hydrolysis of the product – Appendix B7.

4.3.4 SYNTHESIS OF STANDARD DICARBOXYLIC ACYLOXYLACTONES (VIA 4.3.2).

The appropriate diacid dichloride (5 mmol) was stirred with diethyl ether (30 ml, sodium dried). The prepared β -hydroxylactone (0.510 g) was added to the resulting solution. The mixture was left stirring overnight at room temperature and the resulting clear liquid heated to reflux for 2-3 hours. When cooled the mixture was rotary evaporated and the oily residue hydrolysed by standing open to air, overnight. Washing then purified the hydrolysed product, as follows.

The hydrolysed product was dissolved in approximately 15 ml of distilled water to which 1 g of NaHCO_3 had been added. This was washed with diethyl ether (2x10 ml). The aqueous layer was acidified, using 1M HCl , and extracted with dichloromethane (3 x 10 ml). The organic layer was dried (MgSO_4), filtered and rotary evaporated.

The method as described was applied to the synthesis of (a) succinyl (C_6), (b) adipyl (C_8) and (c) suberyl (C_{10}) lactones.

(A) SUCCINYL LACTONE

IR: 2400-3000 cm^{-1} (acid OH stretch); 1600-1800 cm^{-1} ($\text{C}=\text{O}$) contained 1790 cm^{-1} (lactone), 1730 cm^{-1} (ester), 1690 cm^{-1} (acid) – Appendix B8.

^1H NMR, 90 MHz, CD_3OH : δ 2.3 - 3 ppm (dd, $\text{H}_{a,b}$), 2.6 ppm (2 x t, CH_2CH_2), 4.4 ppm (dd, $\text{H}_{c,d}$), 5 ppm (m, lactone ring, CH), 5.5 or > (s, COOH, a broad signal)

- Appendix B9.

FAB, glycerol: m/z 203 (13%, $[\text{M} + \text{H}]^+$), m/z 185 (28%, $[\text{C}_4\text{H}_5\text{O}_2]\text{OCOCH}_2\text{CH}_2\text{CO}^+$), m/z 129 (15%, $[\text{C}_4\text{H}_5\text{O}_2]\text{OCO}^+$), m/z 101 (47%), $\text{HOOCCH}_2\text{CH}_2\text{CO}^+$, m/z 85 $[\text{C}_4\text{H}_5\text{O}_2]^+$, m/z 287 ($[\text{M} + \text{H}]^+$ diester) – Appendix B10.

GC retention time (temperature program (1.5.1), Carlo Erba, BP5) 11.45 min.

(B) ADIPYL LACTONE

IR: 3800-2300 cm^{-1} (acid OH stretch), carbonyl region ($\text{C}=\text{O}$, 1800-1700 cm^{-1}) contains lactone (1790 cm^{-1}), ester (1740 cm^{-1}), acid (1700 cm^{-1}) – Appendix B11.

^1H NMR, 90 MHz, CD_3OD (Appendix B6): δ 0.8 - 1.5 ppm (t, CH_2_{b-e}), 2.3 - 3 ppm (dd, $\text{H}_{a,b}$), 2.3 ppm (2 x t, $\text{CH}_2_{a,f}$), 4.4 ppm (dd, 2 x H), 5 ppm (m, lactone ring), 5.5 or > (s, COOH) Appendix B12 this appendix also includes the ^{13}C NMR spectrum.

GC retention time (temperature program (1.5.1), Carlo Erba, BP5) of the major peak 17.21 min.

(C) SUBERYL LACTONE

IR: 3800-2300 cm^{-1} (acid OH stretch), carbonyl region ($\text{C}=\text{O}$, 1800-1700 cm^{-1}) contains lactone (1790 cm^{-1}), ester (1740 cm^{-1}), acid (1700 cm^{-1}).

FAB⁺, glycerol: m/z 259 (19%, $[\text{M} + \text{H}]^+$), m/z 241 (22%, $[\text{M} + \text{H}]^+ - \text{H}_2\text{O}$), m/z 175 (20%, $\text{HOOC}(\text{CH}_2)_6\text{COOH}_2^+$), m/z 157 (42%, $\text{HOOC}(\text{CH}_2)_6\text{CO}^+$), m/z 343 ($[\text{M} + \text{H}]^+$ diester), glycerol adducts of diacid, lactone, m/z 267, 351.

FAB⁻, glycerol: m/z 173 (100%, $[\text{M} - \text{H}]^-$ suberic acid), m/z 257 (5%,

[M - H]⁻. Both FAB Spectra are included as Appendix B13

4.3.5 SYNTHESIS OF DICARBOXYLIC ACYLCARNITINES (METHOD 1).

Recrystallized trichloroacetic acid (9.6g) was heated to 60°C. The relevant diacid dichloride (10 mmol) and 1g of dl-carnitine hydrochloride were added and the mixture was heated to 80°C for 3 - 4 hours. When cooled diethyl ether (75 ml) was added, dropwise until precipitation began. The solid was then filtered, and the precipitate, a white sticky solid, was washed with diethyl ether and dried.

The product, from above, was then dissolved in hot propan-2-ol (15 ml) and gravity filtered. The filtrate was then added dropwise to diethyl ether (100 ml) and the precipitate, which formed immediately, recovered by filtration and dried.

The above method was applied to the synthesis of succinyl-, adipyl- and suberylcarnitines and spectra included in Appendix B are ¹³C NMR of Succinylcarnitine (Appendix B14), the positive in FAB spectrum of Suberylcarnitine (Appendix B15) and the ¹H NMR spectrum (Appendix B16) of Adipylcarnitine.

4.3.5.1 STANDARD LACTONIZATION REACTION.

The synthesised acylcarnitine (100 mg/l, 200 ml) in acetonitrile was added to 200 µl of phenylbutanoylcarnitine solution (500 mg/l), the internal standard, and dried under a stream of nitrogen. Acetonitrile (400 µl) and 80 µl DPA solution (*N,N*-diisopropylethylamine, 25 µg/ml) were added to the residue in a Pierce Reacti-vial and the vial was shaken for about 30 seconds. The mixture was heated at 125°C for 35 minutes. The sample was cooled to room temperature and dried under a stream of nitrogen. The resultant residue was taken up in 200 µl of ethyl acetate and filtered using a 2 µm filter and a glass syringe. The solution was stored in the freezer until analysed.

4.3.5.2 TMS DERIVATIZATION OF DIACID LACTONE COMPOUNDS.

The diacid lactone (200 μ l, 200 mg/l), prepared via either method, was dried under nitrogen. Acetonitrile (200 μ l) and BSTFA (bis(trimethyl silyl) trifluoroacetamide, 200 μ l) were added to this and the Reacti-vial (Pierce) was shaken for 30 seconds. This was heated for 15 minutes at 70°C. The solution was dried under nitrogen, though not completely evaporated, ethyl acetate (200 μ l) was then added and the solution filtered ready for GC and GC/MS analysis.

4.3.6 SYNTHESIS OF DICARBOXYLIC ACYLCARNITINES (METHOD 2).

4.3.6.1 SYNTHESIS OF THE DIACID MONO-CHLORIDE.

Thionyl chloride (10 mmol) and adipic acid (10 mmol) were added to freshly purified dioxane (20 ml) and refluxed for 5 hours at 80°C, over an oil bath. The resultant clear liquid was then rotary evaporated to yield a white sticky solid. The yield was approximately 1g, 61%.

4.3.6.2 REACTION OF CARNITINE WITH DIACID MONOCHLORIDES.

Carnitine (dl-, 2 mmols) and the diacid mono-chloride (2 mmols) were added to trifluoroacetic acid (2 ml) and heated overnight (16 hours) at 55°C. When cooled to room temperature 10 ml of Analar acetone was added to the carnitine/mono-chloride and this was cooled further to 0°C for 5 hours. This mixture was then added to dry diethyl ether (100 ml) and the white precipitate which formed was filtered, washed with diethyl ether and dried. The yield was 0.10g, 16.2%.

The precipitate was taken up in distilled water (0.5 ml) and washed (3 x 0.5 ml) with diethyl ether. The ether layer was discarded and the aqueous layer freeze dried. The

resultant residue was dissolved in Analar acetone (5 ml) and this was then cooled at 0°C for a further 2.5 hours. The product at this stage was a waxy coating on the sides of the round bottomed flask and was taken up in acetonitrile (500 µl). The standard cyclization procedure was then carried out on the product.

4.3.7. SYNTHESIS OF METHYL ADIPYL CARNITINE.

The mono methyl ester of adipic acid (10 mmol) and thionyl chloride (SOCl₂, 1.19 g, 0.73 ml, 10 mmol) were heated to 80°C for 5 hours, in sodium-dried dioxane. This solution was rotary evaporated and the acid chloride of methyl adipate was weighed.

The product from above (2 mmol) was then heated with dl-carnitine.HCl (0.395 g, 2 mmol) in a few millilitres of trifluoroacetic acid (TFA) for a further 16 hours at 55°C. Analar acetone (10 ml) was added to this when cooled and was stirred at 0°C for 5 hours. The acetone mixture was then added to dried diethyl ether (100 ml), the resultant precipitate being filtered and weighed.

4.3.8 INVESTIGATION INTO FACTORS EFFECTING CYCLIZATION.

4.3.8.1 EFFECT OF A FREE ACID GROUP.

A number of combinations of the solutions octanoylcarnitine, ammonium acetate and the synthesised methyl ester were used to produce both control and reaction samples (Table 4.2). Using 200 µl of each of the reagents, the samples were cyclized in the usual manner and where the volume of the reagents was more than 200 µl the sample was dried under nitrogen and the residue re dissolved in 200 µl of dry acetonitrile before cyclization. Analysis of the products of the cyclization step was by GC and GC/MS.

TABLE 4.2. Samples prepared to investigate the effect of a free acid group on cyclization.

	SAMPLE CONTENT
1	Methyl Ester
2	Methyl Ester, Ammonium Acetate, Octanoylcarnitine
3	Methyl Ester, Ammonium Acetate
4	Octanoylcarnitine, Ammonium Acetate
5	Octanoylcarnitine
6	Octanoylcarnitine, Methyl Ester

4.3.8.2 EFFECT OF SAMPLE CONCENTRATION.

The concentrations of reagents in this experiment were approximately equimolar (25 mg/l) and 200 μ l of each reagent was used when preparing the samples. In this experiment the largest volume used was 600 μ l, (Table 4.3) this was chosen as no great change in the ratio of peak areas in the samples with a total volumes of 600 μ l were seen in experiment.

TABLE 4.3. Samples prepared to investigate the effect of sample concentration on cyclization.

	Sample	Reagent concentration	Amt. each reagent used
1	Methyl ester, Octanoyl carnitine.	500 mg/l	200 μ l
2	Methyl ester, Octanoyl carnitine	500 mg/l	100 μ l
3	Methyl ester, Octanoyl carnitine.	500 mg/l	50 μ l

4.3.9 SYNTHESIS OF THE INTERNAL STANDARD (4-PHENYLBUTANOYLCARNITINE).

4-Phenylbutanoic acid (5 g) and freshly redistilled thionyl chloride (0.367 ml), were heated to 80°C for 3 hours. Carnitine hydrochloride (0.5 g) was dissolved in trichloroacetic acid (2.5 g) and added dropwise through the condenser to the thionyl chloride/phenylbutanoic acid mixture. This was then heated for a further 3.5 hours at 80°C. When the mixture was

cooled diethyl ether (100 ml) was added. The precipitate that formed was filtered, washed with diethyl ether, and dried.

The product, from above, was dissolved in propan-2-ol (approximately 15 ml) and was then filtered. Diethyl ether (50 ml) was added to the filtrate, dropwise. This was then left overnight and the resultant white precipitate was filtered, washed with diethyl ether and dried.

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CHAPTER 5

EXTRACTION AND ANALYSIS OF ACYLCARNITINES FROM DRIED BLOOD SPOTS.

5.1 INTRODUCTION

The aim of the work described in this chapter was to determine if the method of Lowes and Rose [1] for the cyclization and detection of acylcarnitines from urine samples could also be applied to the cyclization and detection of acylcarnitines extracted from dried blood spots. The medium of blood was considered to be very important due to a number of factors. Firstly it was reported that the range of concentrations of acylcarnitines in blood is more limited and stable than those in urine [2, 3] and thus would provide a more suitable method of obtaining a snapshot of the metabolic state at the time of sampling. Also, in the form of Guthrie cards (filter paper cards), blood spots are collected from most babies in the developed world and are therefore more easily obtained than other biological fluids, including urine. Blood spots are collected from a heel prick during the first weeks of life, these spots are then routinely used in hospitals for a range of tests on the new-born including phenylketouria (PKU), organic acid and amino acid analyses. A number of these blood spots are collected, usually four, and therefore the method which will be described has a number of advantages over conventional forms of blood and blood products. Samples are readily available and the Guthrie cards can be stored for a number of years at room temperature. This store of samples means that the method can be evaluated using pre-diagnosed samples thus demonstrating its application prior to the analysis of blind clinical samples. In our laboratory sample viability has been demonstrated through the use of blood spots spiked with acylcarnitines up to sixteen months after preparation and elsewhere the use of dried blood spots has been reported after more than three years [4]. The appearance of the blood spots changed on drying from the bright red expected of fresh blood to brown but was not visibly altered once the drying of the Guthrie spot was completed. The colour of the blood spots when dry was the same as that of clinical blood spots obtained from hospitals.

5.2 RESULTS AND DISCUSSION

The first objective of the work discussed in this chapter was to determine if acylcarnitines could be extracted from blood spots. The blood spots used in the experiments described are prepared from whole blood and stored in dried form on Guthrie cards. As with the analysis of urine samples a second objective was to ensure that the method developed could be set up in a hospital laboratory with equipment which was already available or at a lower cost than the instrumentation presently used for such analyses. Analysis of acylcarnitines extracted from dried Guthrie blood spots was carried out by gas chromatography alone (Carlo Erba -Section 2.1) or GC/MS using an ion trap (Finnigan MAT ITD 800A – Section 2.3) or quadrupole systems (VG 20-250). Analysis was carried out on spiked standards and, later, the clinical applications of the method were explored.

In developing this methodology there were a number of areas of potential problem to consider. The first was the matrix itself, which by its very nature is a complex system. A second was the selectivity both in terms of the extraction of acylcarnitines and later of their detection. The former would be assessed during the method development and from the experience gained in the analysis of acylcarnitines from other biological matrices. The latter would also rely on experience in a detailed study of the fragmentation patterns of acylcarnitines and their lactones. Whilst this method should be considered qualitative due to the low levels of acylcarnitines reported in blood [4] this factor also necessitate a low limit of detection for acylcarnitine lactones.

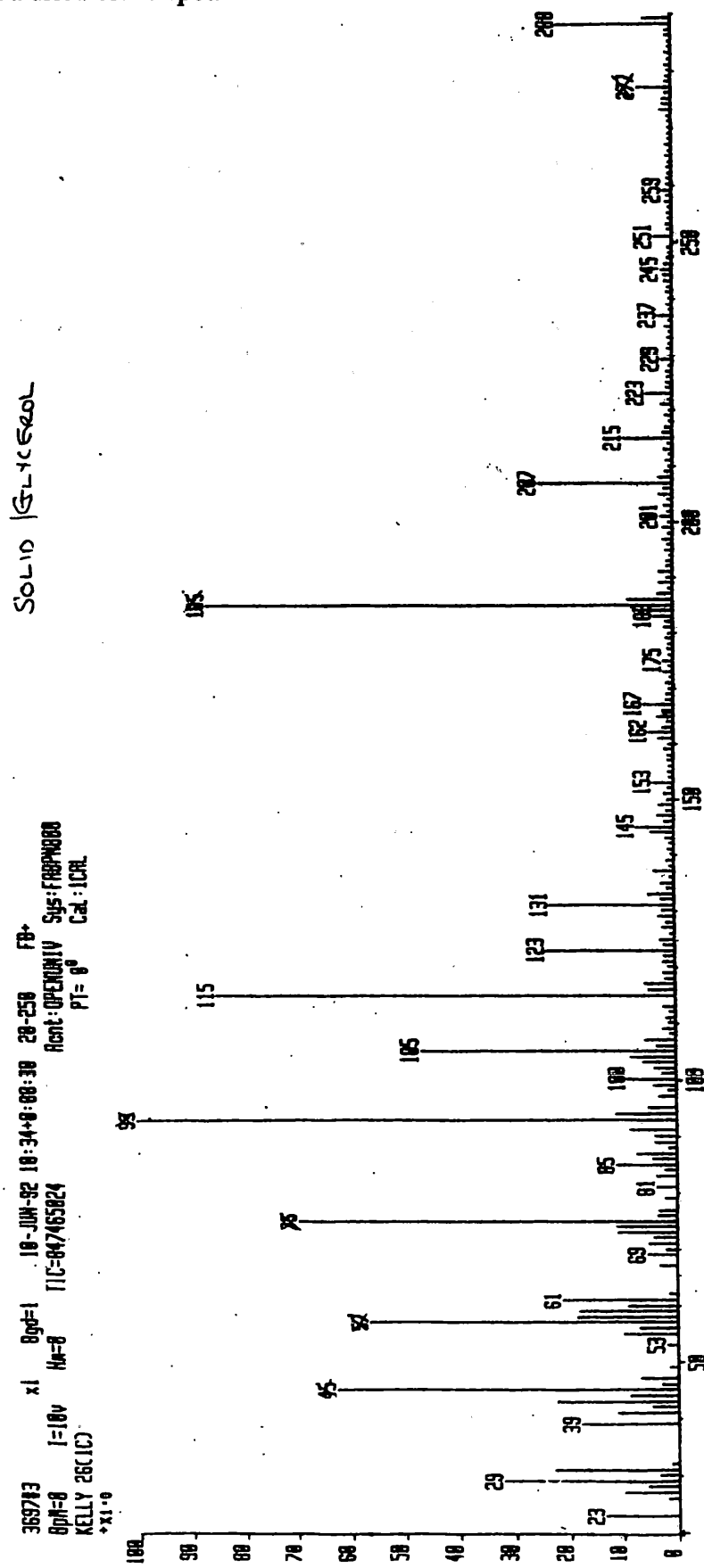
The first stage in the development of this analytical method was to establish that the extraction of acylcarnitines from dried spiked blood spots was possible. Fresh blood was spiked with octanoylcarnitine (Experiment 5.11.2). Octanoylcarnitine was chosen as it had been used in earlier studies both as a standard as in this case and it has also been identified

from clinical urine samples. From these earlier studies the chromatographic retention time of this compound was well established as was the mass spectral information. Also, it is the key compound in the diagnosis of MCADD.

There were a number of possible methods of extraction with the literature suggesting sonication as the most reliable and widely used [5]. The cyclization procedure, which the acylcarnitines undergo has been shown to be sensitive to a number of factors including the concentration of acylcarnitine present and the presence of non-acylcarnitine contamination. Though the primary aim of the extraction was to obtain the highest yield of acylcarnitines from any given blood spot, it was also necessary to consider other co-extracted material which might interfere with the process of cyclization. It had been shown that co-extracted components in the cyclization mixture or a high concentration of acylcarnitines, as in the case of inappropriately made-up standards, can inhibit the reaction. Where high concentrations of acylcarnitines were present polymer formation might result (Lowes, unpublished work) though this would be of greater concern during method development than in the case of analysis of samples where biological levels are much lower.

In order to establish the most suitable method for later cyclization and GC analysis, separate dried blood samples were shaken by hand, sonicated and vortexed in methanol to ascertain the efficiency of each extraction procedure (Experiment 5.11.3). Methanol was solvent chosen for the extraction of acylcarnitines from dried blood spots, prior to analysis using fast atom bombardment coupled with mass spectrometry, as it had been cited as an appropriate extraction solvent in reviewed literature [5]. Analyses were carried out on spiked blood spots made up with solutions of octanoylcarnitine. Aliquots (0.5ml) of whole blood obtained from a healthy male adult were spiked with a known concentration of octanoylcarnitine solution (Experiment 5.11.3, Table 5.1) and this blood mixture was spotted onto Guthrie paper. Fully dry areas (6 mm) were punched from the card and

Figure 5.1 The FAB⁺ spectrum of octanoylcarnitine after extraction (methanol) from a dried blood spot.



extracted twice into methanol (Experiment 5.11.3). The aliquots of methanol were then combined and dried. The result was a brown residue, which should contain octanoylcarnitine but, due to its pigmentation, clearly contained co-extracted material (i.e. haemoglobin).

TABLE 5.1. – Spiking and preparation of standard blood spots

Cone. octanoylcarn. (mg/ml)	Vol Octanoylcarn. used/ 0.5ml aliquot (μ l)	Amt. octanoylcarn./ blood spot (ng)
5.0	200 μ l	125
5.0	100 μ l	62.5
5.0	75 μ l	46.9
5.0	50 μ l	31.2
5.0	25 μ l	15.6
0.5	100 μ l	6.25
0.5	50 μ l	3.12
0.5	20 μ l	1.25
0.5	2 μ l	0.125
blank	-	-

To ensure that the required acylcarnitine was present in the residue FAB/MS analysis was carried out on the residue. A small amount of the residue, from the extracted spiked blood spot, was removed and added to a few microlitres of glycerol (the solvent matrix for FAB analysis) on the tip of the FAB probe. FAB analysis was carried out as described in Chapter 2, Section 2.6 on the VG 20-250 mass spectrometer. Figure 5.1 is the FAB spectrum acquired in positive mode. The presence of octanoylcarnitine was demonstrated with very few non-matrix contamination peaks. In the FAB⁺/MS spectrum of the extraction residue the [M + H]⁺ peak at m/z 288, corresponding to the relative molecular mass of octanoylcarnitine, is one peaks recorded. The small peak at m/z 162 may be attributed to carnitine (due to some carnitine in the original commercial octanoylcarnitine) and some fragmentation of octanoylcarnitine. The other usual fragments at m/z 85 and

144, which are common to this group of compounds are not visible due to the low response and presence of a large number of matrix peaks. Given that the sample appeared impure analysis by FAB did not give information as to their origin, possibly due to poor ionization. Further work was carried out on this sample as the impurities present might have a negative effect on the cyclization and subsequent analysis. These experiments are detailed later in this Experiment 5.11.6 of this Chapter.

Once it was established that extraction using sonication, shaking or vortex mixing was effective, as shown in the FAB identification of acylcarnitines, the cyclization procedure was carried out on the residues. The result on GC/MS analysis was a peak corresponding to octanoylcarnitine lactone; identified by its chromatographic retention time, mass spectrum and an in-house library match. The peak areas recorded suggested sonication as the most efficient means of extracting acylcarnitines from dried blood spots. The peak area recorded for octanoylcarnitine lactone from sonicated, spiked blood spot samples was approximately twice the intensity of the shaken or vortexed samples.

The duration of sonication was investigated (Experiment 5.11.3 ii) in order that it might also be optimized. Dried, spiked blood spots were extracted, with isovalerylcarnitine added to the extraction solvent as an internal standard, and the sonication time of the samples were varied from 2 - 30 min for each millilitre of solvent (as Table 5.2). The peak area of octanoylcarnitine lactone relative to the internal standard recorded in the case of 2 x 10 min sonication was significantly greater than at the other sonication times. A gradual increase in the peak area ratio was observed over the initial increments in sonication time to reach this maximum. After this point the ratio decreases probably due to an increase in co-extracted material hindering the cyclization process. This led to the use of a ten minute sonication for the extraction of acylcarnitines from the blood spots.

Table 5.2 – Extraction conditions versus peak area ratio (octanoylcarnitine/IS) during method optimization. The experiment was carried out in triplicate and the mean values are reported

Work-up	Peak area octanoylcarnitine/IS
Blood spot (15.6ug/blood spot), sonicated 2 x 2min.	0.83
Blood spot (15.6ug/blood spot), sonicated 2 x 5 min.	1.04
Blood spot (15.6ug/blood spot), sonicated 2 x 10 min.	1.43
Blood spot (15.6ug/blood spot), sonicated 2 x 15 min.	0.79
Blood spot (15.6ug/blood spot), sonicated 2 x 20 min.	0.13
Blood spot (15.6ug/blood spot), sonicated 2 x 30 min.	ND

ND – Nil detected

As carnitine and acylcarnitines are found in the cytosol and the mitochondria of *in vivo* samples, it was necessary to determine the efficiency of spiking and preparation of blood spots in mimicking the natural situation. Work was carried out to determine if any chromatographic effect was observed when applying the blood to the paper (Experiment 5.11.4). It was considered that, similar to TLC, a concentrated area of acylcarnitine might occur at the point where the blood was initially spotted or diffusion might occur, concentrating the spike at some distance from the origin. To determine if this effect prevented an even distribution of octanoylcarnitine, a blood spot with a diameter of 6 mm was punched from the within a printed area designating a complete spot. The remaining spot, outside the punched area, which had approximately the same surface area was also removed. Both the inner and the outer areas were worked-up separately using the sonicated methanol extraction. The standard cyclization procedure was then applied to both samples and GC/MS analysis carried out. Peaks were recorded from both the inner and outer blood spot samples. These peaks occurred at a retention time window consistent with octanoylcarnitine in its lactone form, at approximately 11.7 min, using a BP5 GC column and under the conditions described in Chapter 2, Section 2.1. In the EI mode the presence of octanoylcarnitine lactone was further confirmed by the fragments at m/z 85 (100%), 144 (10%) which have been described previously as characteristic for acylcarnitines. In the CI mode, with isobutane, the protonated molecule was the base peak

of the spectrum at m/z 229 and fragments at m/z 85 (72%) and 125 (15%) which are again characteristic for this group of compounds were also detected. No significant differences were recorded in the chromatographic peak areas between the inner and outer areas of the punched spot.

Aqueous octanoylcarnitine solution was added directly to Guthrie paper in order to establish if, without the blood matrix, any chromatography occurred. Areas of Guthrie cards were then prepared, with the inner and outer areas of card approximately the same area, and the paper analysed as above. The process of chromatography had evidently taken place in this experiment, with almost four times more octanoylcarnitine lactone detected from the outer section of the printed spot, leading to the hypothesis that it is the matrix of the blood which binds the octanoylcarnitine preventing chromatographic separation of the octanoylcarnitine component. It is therefore assumed from this point that acylcarnitines would be evenly dispersed through both clinical and spiked blood spots.

It has therefore been demonstrated that the preparation and spiking of blood spots used in the experiments outlined in this thesis does mimic the situation *in vivo* and that sonication of these samples for 2 x 10 min is the most effective extraction method. Samples derived from dried blood spots, described in this thesis, are prepared in the manner described (Experiment 5.11.2), extracted via sonication (Experiment 5.11.3), and cyclized unless otherwise stated.

The sample work-up procedure was then performed on blood spots spiked with octanoylcarnitine in the same concentrations as used in previous experiments (Table 5.1). These samples produced very low responses for octanoylcarnitine lactone, on analysis by GC and GC/ITD in the EI mode. Identification of the acylcarnitine was through its retention time (approximately 11.7 min) and trace levels of key fragments at m/z 85 and

144. Even from blood spots spiked with very high concentrations of octanoylcarnitine (125 µg, 62.5 µg) the peak areas observed were lower than expected, when compared to results from the analysis of aqueous standards, leading to the proposal that methanol alone might not be the most suitable solvent for extraction. It was therefore necessary to determine the factor(s) contributing to this low yield. There were two areas where potential problems could exist. The first was the solvent extraction phase of the work-up and the second the cyclization of the sample. Initially the latter was investigated.

A number of Guthrie spots spiked with octanoylcarnitine solution (as Table 5.1) were subjected to sonicated methanol extraction and the resulting residues containing the acylcarnitine and any other co-extracted materials were sent to Dr.S.Lowes (VG Bio Tech, Altrincham, UK.), Experiment 5.11.5. It was hoped to determine if mass spectrometry by electrospray ionization (ESI/MS) was a viable analytical technique for the analysis of acylcarnitines from this medium (see Chapter 6). This technique would be an exciting avenue of exploration towards the detection of acylcarnitines as derivatization would not be necessary. The initial analysis of a loop injection of the extraction residue dissolved in 100 µl of a chloroform/methanol mixture was carried out to determine if the residue provided contained acylcarnitine at levels suitable for analysis or if the extraction process facilitated the extraction of other molecules which would interfere with this process. It was also hoped to quantify the levels of octanoylcarnitine obtained.

On analysis of the extraction residue by electrospray mass spectrometry it was shown that there was considerable protein contamination of the samples with the detection of a number of high relative molecular mass molecules carrying the multiple charges characteristic of this analytical technique. With this method of analysis a spray of analyte and solvent from a probe is dispersed into a highly charged field, sample molecules can then take up charges thus lowering its mass to charge ratio and rendering larger molecules

such as proteins within the range of the detector. Electrospray has been optimized for the detection of proteins and in the samples from the extraction of spiked blood, proteins were detected. Haemoglobin was the major constituent as predicted from the colour of the residue. Octanoylcarnitine could not be detected among this considerable chemical background.

The presence of high levels of protein could have a number of effects on the analysis of acylcarnitines by GC, via the method proposed. The reaction to form the lactone ring system from an acylcarnitine would be affected by the high level of proteins observed due to the sensitivity of the cyclization to contamination as described earlier. These compounds would not pass through the column of the GC and therefore a second problem would be contamination of the injector area from the analysis of a large number of samples of this nature, thus inhibiting routine application of this method.

Continuous flow fast atom bombardment (FAB) has been successfully used in the field of large scale clinical acylcarnitine analysis [6]. With this technique analytes take on a single extra charge so large protein molecules like haemoglobin are beyond the detected mass range. This would explain the lack of reports of protein contamination when samples are prepared using this method for analysis by FAB/MS. If proteins were extracted into all samples previously prepared, the susceptibility of the lactonization reaction to impurities would suggest interference with the cyclization of the acylcarnitines, therefore accounting for the low yields observed.

In summary, the methanol may extract acylcarnitines very efficiently from blood spots but co-extracted materials inhibit further analysis by cyclization and GC/MS, and by electrospray mass spectrometry. The selectivity of the extraction solvent needed to be addressed.

A series of experiments (Experiment 5.11.6) were designed to determine if an alternative solvent, or mixture of solvents, could be found which facilitated the extraction of octanoylcarnitine, without that of proteins and/or other compounds, which appear to inhibit the cyclization and analysis.

A number of single solvent systems were used in an attempt to improve the yield of lactones for analysis (Table 5.5). Pentan-2-ol, butan-1-ol and hexan-2-ol were used for the extraction of acylcarnitines as they had already been proven successful at varying pH values in the extraction differing chain-length acylcarnitines from urine samples [7]. On analysis by GC/EIMS using an ion-trap, the use of these solvents provided recoveries that were similar to, or worse than, that obtained following extraction with methanol and cyclization of spiked blood spots. Hexan-2-ol demonstrated a greater chromatographic signal-to-noise ratio than had been detected on analysis of methanol extracted blood spots, with pentan-2-ol providing the poorest detection of octanoylcarnitine lactone. Pentan-2-ol and hexan-2-ol extracts on freeze-drying were the least haem-coloured in appearance, with the butan-1-ol sample having an increased coloration but not to the level recorded with methanol. This lack of colour in the extracted residues, combined with the poor detection levels in these particular samples would suggest that the disruption of the cells, which causes the release of the haemoglobin (and possibly other protein and material) and the freeze-dried residue pigmentation, is also responsible for the release of the acylcarnitines from the blood spot. The results of this experiment may also indicate that the method used to prepare the spiked blood spot was effective in mixing of the octanoylcarnitine intimately with the blood components.

Literature reviews including a paper by Masaru *et al* [8] suggested that the protein could be immobilised on the Guthrie card but the acylcarnitine content still extracted by using a

mixed solvent system. Methanol would facilitate the extraction and a second solvent known to precipitate proteins could be included thus removing or reducing protein interference within the later lactonization. The suggested second solvents were acetone, ethanol or chloroform, all of which were used in conjunction with methanol. Mixed solvent systems were used with varying volumes of methanol as one component and the solvents suggested to immobilise the proteins as the second (Table 5.6). Blood spots with octanoylcarnitine (6.25 µg) spiked at a constant level were used for these extractions. In all cases the required acylcarnitine was detected and characterized by the retention time, which was very reproducible, and the presence of characteristic fragments, with m/z 85 usually as the base peak and m/z 144 with a relative intensity of between 5 and 15%. Peaks corresponding to the molecular ion (m/z 228) or the protonated molecule (m/z 229) due to self-chemical ionization, as seen in samples at higher concentrations were not detected with any of the solvent systems at this lower concentration.

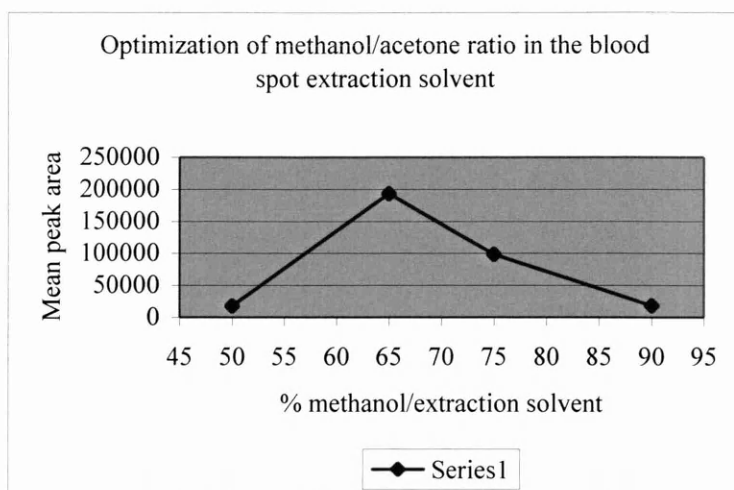
TABLE 5.3 – (a) Optimisation of extraction solvent while (b) represents a graphical review of this data.

(a)

	Methanol / Acetone / Ethanol (%)				
	90/10/0	75/25/0	65/35/0	50/50/0	0/0/100
Peak Area	21767	96947	204226	182048	47050
	15725	87875	175241	149396	SL
	17068	110164	199795	192614	37607
Number of replicates	3	3	3	3	2
Mean area	18187	98329	193087	174686	42329
Standard deviation	3173	11209	15613	22530	
Coefficient of variation (%)	17.44	11.39	8.09	12.90	

- Peak areas - levels of octanoylcarnitine lactone/sample on analysis of a standard volume by GC/ITD.
- All samples were prepared in triplicate.
- SL – sample lost during sample preparation, two samples only therefore no statistics included.

(b)



The use of varying concentrations of Analar acetone or ethanol in methanol as extraction solvents gave residue pigmentation with an appearance and intensity like that observed with methanol. Analysis by GC/ITD clearly showed that both solvent mixture systems produced a significantly greater yield of the octanoylcarnitine lactone than the pure methanol system. An attempt was then considered to optimize the solvent ratio for the extraction procedure. As methanol (100%) had not proved successful it was decided to begin at the other end of the scale and concentrations from 100% acetone (i.e. 2 x 1ml) to 10%:90% methanol:acetone.

The peak area was considered to be the most reliable quantitative indication of the level of octanoylcarnitine lactone formed in the samples. The results from analysis of five samples extracted and analysed in triplicate of varying concentrations of methanol and acetone are shown above (Table 5.3a). The data for the analysis of the pure methanol extraction was also recorded and was significantly lower than the maximum obtained with the mixed solvent (acetone/ methanol). The results of this set of experiments are illustrated graphically above (5.3b) and clearly show the optimum methanol content of the extraction solvent system as 65%. A comparison was then carried out between this preferred

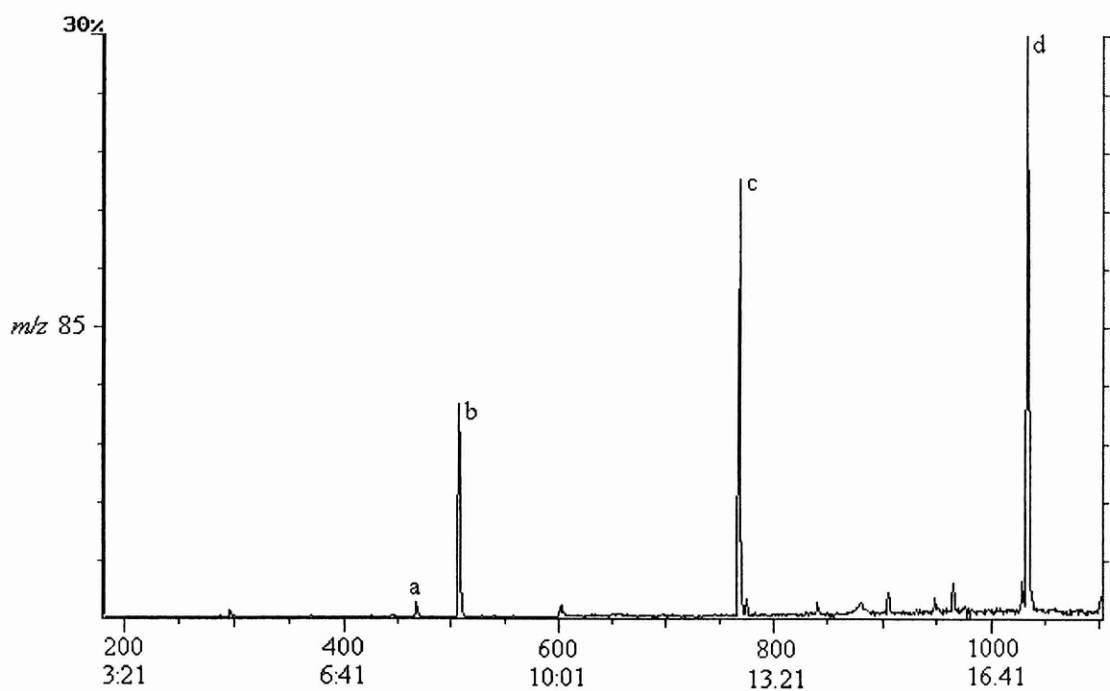


Figure 5.2 Mass chromaogram of m/z 85 for blood spot spiked with (a) butanoylcarnitine, (b) isovalerylcarnitine, (c) octanoylcarnitine and (d) dodecanoylcarnitine. This illustrates the extension of the extraction and lactonisation method to acylcarnitines of differing chain lengths and configurations.

acetone/methanol system and a solvent system of chloroform/methanol in a 2:1 ratio, as used [8] for the analysis of acylcarnitines from urine. This solvent mixture was used as it had been shown using electrospray that there were a number of co-extracted proteins which might have hindered the cyclization process. The presence of chloroform in this mixture to immobilize proteins [8] and its more effective in the extraction of octanoylcarnitine from the prepared dried blood spots made this the extraction solvent mix of choice. A solvent mixture of chloroform and methanol in a two to one ratio was used in the preparation of any further extracted samples with each spot sonicated (2 x 10 min/ml).

Although octanoylcarnitine is quite representative of the medium-chain acylcarnitines that one would expect to detect from a clinical blood spot of a patient with MCADD or MADD, acylcarnitines in nature occur with a wide variety of chain lengths and configurations. To determine if the method described here was applicable to short and long as well as the medium-chain lengths and also those of branched chain configurations, fresh blood was spiked with equimolar quantities of a number of acylcarnitines (equivalent to those for octanoylcarnitine in Table 5.1). These acylcarnitines were butanoylcarnitine, dl-hexanoylcarnitine chloride, dl-octanoylcarnitine chloride and dl-dodecanoylcarnitine chloride, the former synthesised for these experiments [1, 9] and the other three commercially obtained (Experiment 5.11.9). A second mix was prepared containing butanoylcarnitine, isovalerylcarnitine, octanoylcarnitine and dodecanoylcarnitine lactones, as shown in Figure 5.2.

In these samples the peaks corresponding to the acylcarnitine lactones of all four compounds were detected in samples of high spiked concentration. Figure 5.2 depicts a mass chromatogram of m/z 85 for one of these samples. The peaks listed as (a) -(d) could be detected in the total ion chromatogram at higher concentrations although (a) was close to the detection limit. All lactones were detected using the mass chromatogram of m/z 85

which is assigned to fragmentation to the lactone ring (the base peak in most of the spectra obtained) and m/z 144 (2 - 20%) a second diagnostic fragment for acylcarnitines. The molecular ions of the acylcarnitine lactones, M^{++} at m/z 172 (butanoylcarnitine lactone), 186 (isovalerylcarnitine lactone), 228 (octanoylcarnitine lactone) and 284 (dodecanoylcarnitine lactone) were not detected, although some self-CI of the acyloxylactones was observed at higher concentrations. In samples of lower acylcarnitine concentrations, butanoylcarnitine lactone was not observed because it fell below the limit of detection. This fact and the relative peak areas of the lactones in Figure 5.2 would suggest that whereas the solvent system (chloroform/methanol, 2:1) had been optimized for the extraction of medium to long-chain acylcarnitines, further modifications would be necessary to extract short-chain acylcarnitines efficiently. A more polar system might be more suitable for the extraction of the shorter or branched-chain acylcarnitines.

Though the method developed for the extraction and analysis of acylcarnitine from dried blood spots has been shown as effective, modifications have been considered to establish if the time required to work-up the samples could be shortened. Any preparation towards the analysis of both spiked and clinical blood spots should be as straight-forward as possible and therefore ideally the use of a single solvent would be preferred to a solvent mixture. Acetonitrile was an obvious choice for the solvent extraction of acylcarnitines allowing the extraction to run smoothly into the cyclization with minimal disturbance or transfer of the reagents since the reaction is also effected in acetonitrile. Extraction of acylcarnitines using acetonitrile alone was therefore considered. Blood spots spiked with the medium-chain acylcarnitine, octanoylcarnitine, were sonicated (2 x 10 min) in acetonitrile and the solvent aliquots pooled and dried. The residue was lactonized in the usual manner and GC analysis performed. No acylcarnitines were detected via this procedure either by detection using the total ion chromatogram or selected ion retrieval at m/z 85, which would be the expected base peak from the C_8 acyloxylactone and therefore give the optimum response.

Further studies were undertaken to investigate the potential for cyclization of acylcarnitines from dried blood spots without any prior extraction. To achieve this end a blood spot containing 6.25 µg of octanoylcarnitine was added directly to a Reacti-vial containing the cyclization reagents, acetonitrile (200 µl) and *N,N*-diisopropylethylamine (80 µl, 25 µl/ml solution) and these were lactonized as normal at 125°C. This initial sample suggested that the direct cyclization was effective. Octanoylcarnitine lactone was detected and characterized with a retention time in the expected region and the familiar mass spectral pattern. Samples were then prepared from standard blood spots (as Table 5.1) and they demonstrated that acylcarnitines could be extracted and cyclized in a single step with yields of lactones similar to those with conventional solvent extraction with methanol: chloroform. These experiments show that acylcarnitines are extracted from dried blood spots into hot acetonitrile (but not into cold acetonitrile), or that acylcarnitines are cyclized in the blood matrix and the lactone products dissolved in the solvent. Further studies of clinical samples have not demonstrated an improvement on analysis. A quantitative comparison of the extraction and direct cyclization methods is presented within Section 5.5 of this thesis.

A number of samples were prepared to test the feasibility of shortening the cyclization step in the work up of acylcarnitines from biological fluids, primarily blood spots. A study was undertaken to determine the effects of microwaves to allow the reaction time to be cut to minutes or even seconds (Experiment 5.11.7). Normal reaction heating conditions for the cyclization step were thirty-five minutes at 125°C in a heating block with acetonitrile as the solvent and DPEA solution present.

In this study a known concentration of octanoylcarnitine was cyclized in the normal manner as a control sample. Samples of identical octanoylcarnitine concentration were prepared and these were subjected to a variety of microwave intensities and durations (Table 5.7, Experiment 5.11.7, page 159). The lactonization reagents used were the standard volumes and concentrations. There was little or no sign of any cyclization in the earlier samples (Table 5.7, 1-5, Experiment 5.11.7 i, page 159) with all samples analysed by GC alone and monitored using retention time relative to standard octanoylcarnitine lactone and peak areas. In later samples a peak was detected corresponding to the retention time of the lactone from octanoylcarnitine (11.7 min) though these were of lower peak areas than would be expected using the conventional heating method. The extent of cyclization recorded is likely to reflect the heat generated during the microwave procedure rather than a direct effect of the microwaves, as the Reacti-vials when removed from the oven were hot to touch. Normally, the cyclization is brought about by the application of heat so some lactonization would be anticipated as the microwaves warm the solution. When the reaction had reached time two minutes (Sample 10, Table 5.7, page 159) it was estimated that the upper limit of microwave effect was being reached and that temperature was now the major factor in any cyclization recorded.

No further work has been carried out into the effect of microwaves on the cyclization of acylcarnitines as the levels of detection of the octanoylcarnitine lactone were approximately 5 times lower than that normally achieved. It is unlikely that the time saved by implementation of either of a microwave method would merit the decrease in peak area observed for the octanoylcarnitine lactone.

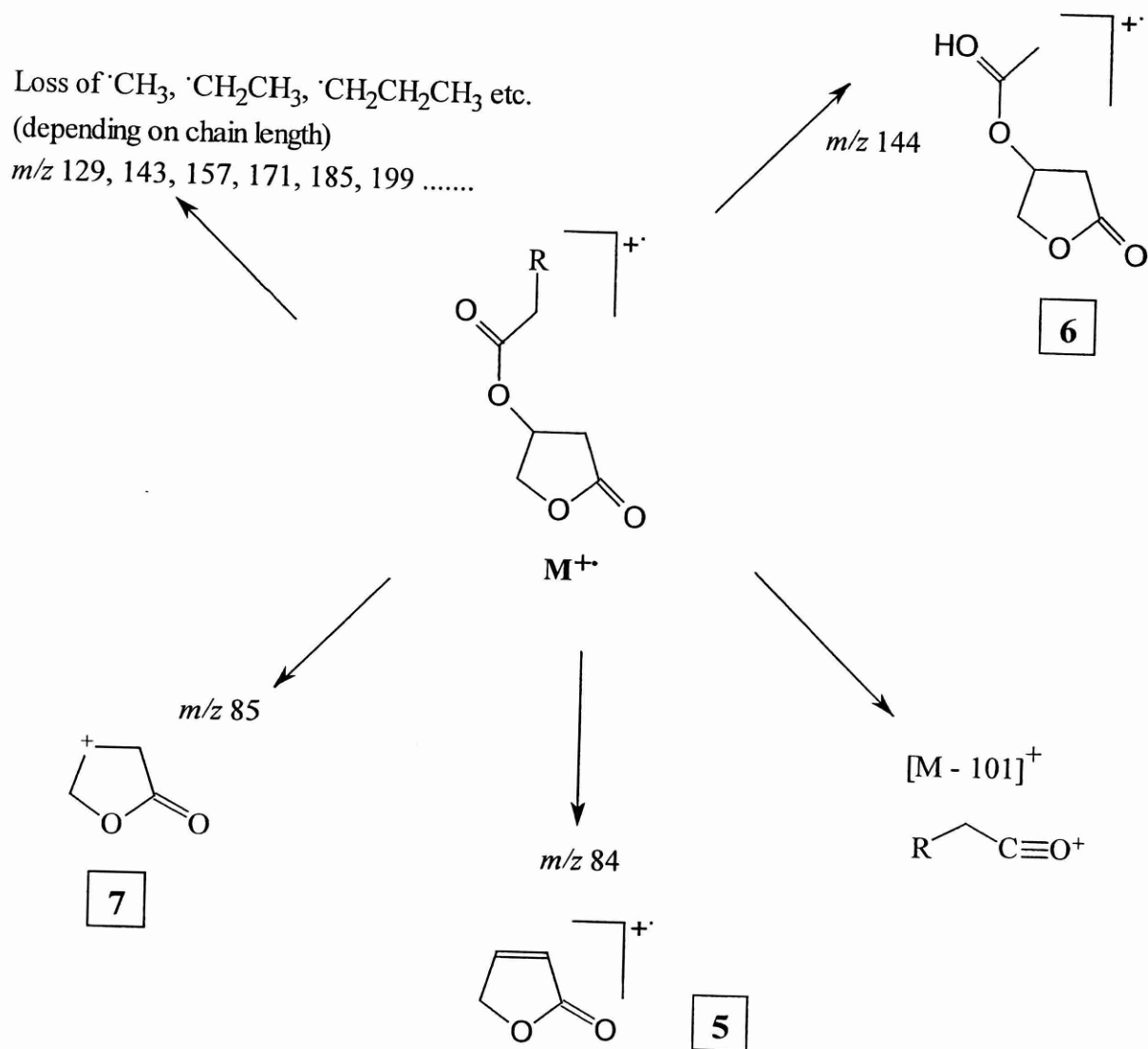


Figure 5.3 Schematic of the key fragments associated with the characterisation of acylcarnitine lactones

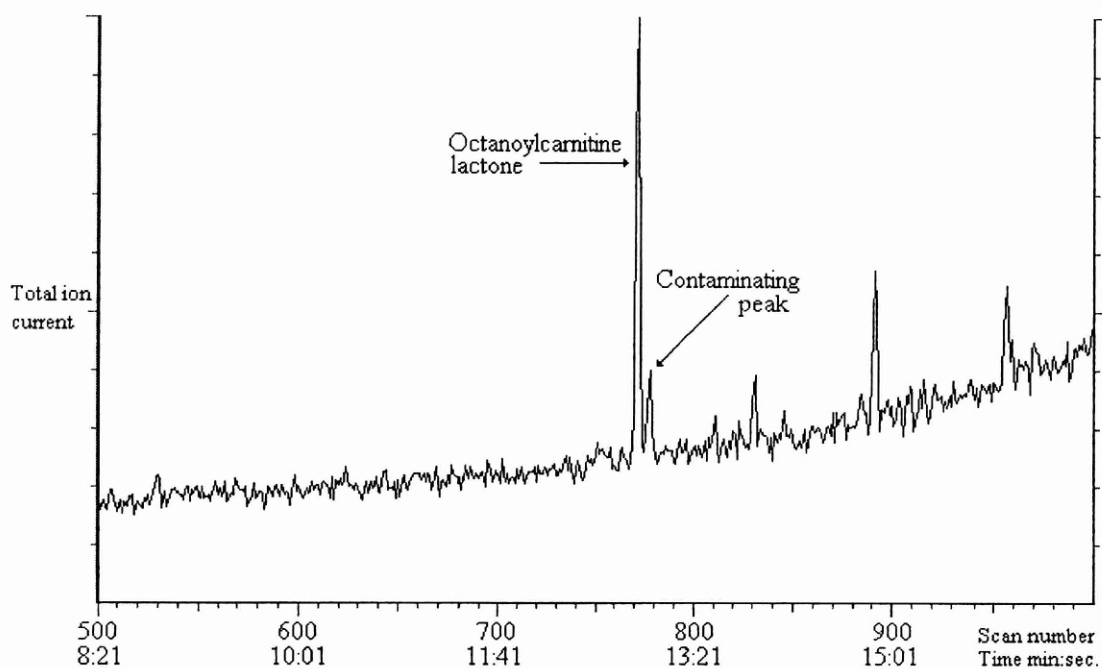
5.3 SAMPLE CONTAMINATION - AN INVESTIGATION

It has been established that using a BP5 GC capillary column and with the temperature program (as described in Section 2.1) octanoylcarnitine, in its derivatized lactone form, would elute with a retention time of just under 12 min and could be characterised through the detection of key fragment ions (Figure 5.3).

A number of spiked blood spots of varying concentrations (Table 5.1) were prepared by methanol extraction and analysed using gas chromatography without coupling to a mass spectrometer. This set of analyses gave rise not to the expected single peak, in the retention time window of interest, but to two very closely eluting peaks. The full chromatogram contained four major peaks at retention times of 3.73 min, 11.73 min, 11.81 min and 13.91 min. The first of these peaks (3.73 min) has been assigned to octanoic acid, which has a relative molecular mass of 174, following analysis of a commercially obtained sample of this acid. There are a number of possible sources of this compound and these include its presence in the original purchased octanoylcarnitine or some breakdown of the acylcarnitine either during cyclization or analysis. The later peak at 13.9 minutes has been identified as a phthalate plasticiser due to its sharp peak shape and the presence of a base peak fragment at m/z 149 which is characteristic of this group of compounds. The retention time of octanoylcarnitine lactone was previously recorded at approximately 11.7 min. The largest peak in the first recorded chromatogram was that of 11.73 minutes and was tentatively assigned to octanoylcarnitine lactone on the basis of the retention time, which had been shown as very reproducible, and its variation in peak area relative to the 11.91 min as the levels of octanoylcarnitine in the spiked blood spots altered.

The chromatograms in Figure 5.4, overleaf, later obtained by GC/MS (Finnigan MAT ITD 800A), illustrate peak areas of interest in a ratio of approximately 4:1 (Fig 5.4 (a)),

(a) Octanoylcarnitine spiked blood spot,
Methanol extraction.



(b) Spiked blood spot, methanol extraction.

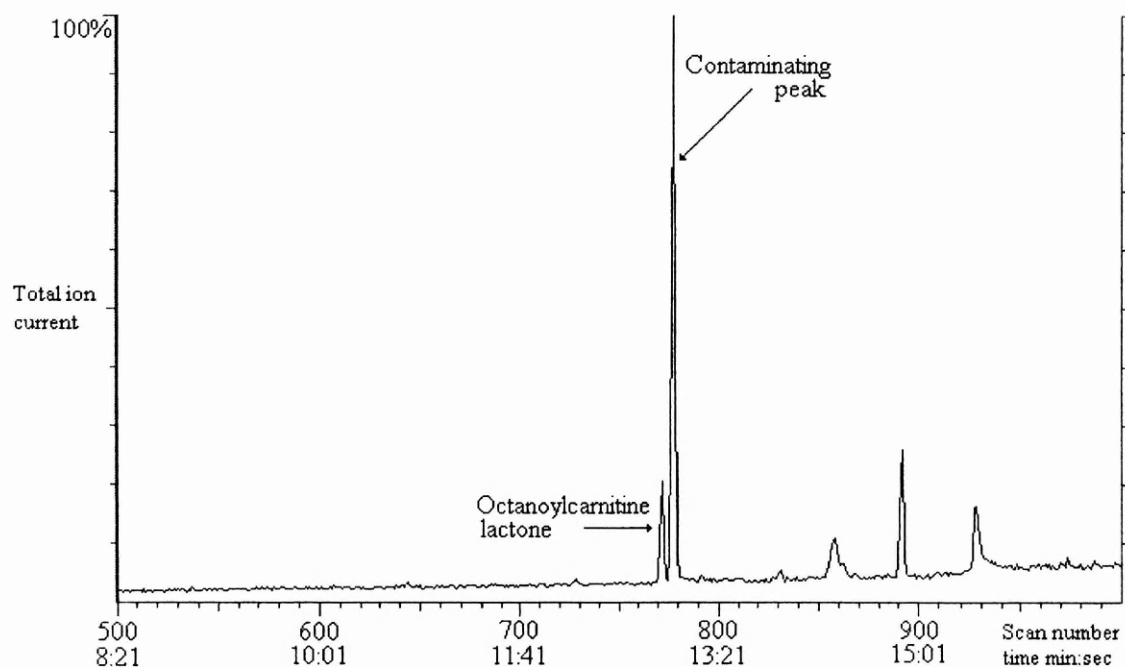


Figure 5.4 Octanoylcarnitine lactone at two concentrations (a) 125ug/blood spot
(b) 15.6ug/blood spot. Both are illustrated relative to the contaminant peak.

octanoylcarnitine lactone (11.73 min): contaminant (11.81 min). This chromatogram was from a blood spot spiked with a high concentration of octanoylcarnitine. The variation in relative peak areas can be clearly seen in the second chromatogram (Fig 5.4 (b)), from a blood spot with lower concentration of spiked octanoylcarnitine, where it can be seen that a reversal of the observed ratio has taken place. The former chromatogram was obtained from a blood spot spiked with approximately 125 µg of octanoylcarnitine while the latter had approximately 15.6 µg of octanoylcarnitine. This variation over a range of octanoylcarnitine spiked blood spots added strength to the assignment of octanoylcarnitine lactone (11.73 min) with little variation in the peak area of the second component. The retention times discussed in the text refer to the initial sample GC times rather than those recorded using the ITD in this example.

In an attempt to identify the additional peak in the chromatographic region of interest the sample was analysed by GC/MS (VG 20-250) with conditions as described in Section 2.3. The chromatographic resolution was very poor even for samples that were heavily spiked with octanoylcarnitine although two peaks could be detected. The mass spectrum of the contaminating peak was obtained but there was no spectral match in the computerised library. This was due both to limitations in the outdated library available and the poor quality mass spectrum presented. In order to identify the peak of interest the Eight Peak Index (RSC) [10] was used to obtain a match manually via comparison of mass spectral data. The index allows comparison based on the abundance of the apparent molecular ion of the compound of interest, together with the relative ion abundance of the eight largest peaks in the EI mode. Using these criteria it was possible to propose that the contaminant was *N*-butylbenzene sulphonamide. The fragmentation of the sulphonamide in the EI mode with a base peak of m/z 77 and significant fragments at m/z 141 (82%), 170 (73%), 51 (45%), 78 (10%), 158 (12%) and 171 (5%) together with some self-CI to give a protonated molecule ion at m/z 214 (5%). GCCI/MS, using the ITD 800A, was used to

lend further weight to the designation of 213 as the relative molecular mass of the compound, with the presence of an $[M + H]^+$ protonated molecule in the CI mode (isobutane) at an m/z value of 214 (100%) with very few fragments present. The contaminant was also confirmed as *N*-butylbenzene sulphonamide after further analysis by GCEI/MS using an ion-trap, which gave good resolution and a successful library match with a newer computerised library. The other peak in the region was confirmed by GC/MS as being the required octanoylcarnitine lactone.

With the contaminating peak identified, as the second of the two peaks in the retention time window of interest, attempts to remove the *N*-butylbenzene sulphonamide from the sample were undertaken.

It was considered that the filter paper onto which the blood was spotted might be the source of the contamination (Experiment 5.11.11.ii). Guthrie cards used in these experiments were obtained from two different sources (Milton Keynes Hospital, Army Medical College). On extraction of blank areas of paper with methanol, both were found to be contaminated with a peak of a retention time (12 min) corresponding to the sulphonamide and to have on GC/EIMS analysis the predicted mass spectral fragmentation pattern as listed in Table 5.4 and depicted in Figure 5.5. In an attempt to remove any contaminants from the paper prior to acylcarnitine extraction, a hexane pre-wash of the spots was added to the work-up. Though a slight reduction in the levels of contamination was recorded it was not significant enough to be incorporated into the standard method or to alleviate the presence of the sulphonamide. Diethyl ether and ethyl acetate were also used as washes for the paper prior to extraction but as in the case of hexane no significant changes in the peak area of the contaminating peak were observed (Table 5.9).

A study was also carried out to determine if the contamination appeared at a particular point during the extraction/cyclization work-up of the samples. Gas chromatographic analysis of the samples was carried out after the extraction step of the procedure and at the end point of the work-up, after cyclization. This set of analyses clearly illustrated that contamination was present from the initial extraction step in the preparation of the dried blood spots.

The nature of sulphonamides as detergents gave rise to the possibility of personal (Experiment 5.11.11 iv) or glassware (Experiment 5.11.11 v) contamination. A number of experiments were carried out in order to eliminate these possibilities. To determine if personal contamination of the blood spots had occurred two areas (6 mm) of filter paper, from the same Guthrie card were punched out. One of the spots was then handled both with and without gloves to maximize the potential for outside and personal contamination. The second spot was not handled but was placed directly in a clean test tube. These two samples were sonicated in methanol and, when finally analysed by capillary gas chromatography coupled with mass spectrometry, shown to contain similar unreduced levels of the sulphonamide. Identification of the sulphonamide was again by chromatographic retention time and mass spectral data. To eliminate the possibility of glassware contamination, the stoppered pyrex test-tubes used for the sonication and freeze-drying of extraction solvents were washed in turn with the extraction solvent itself, as the contaminant was obviously soluble in methanol. This would have removed any sulphonamide from the glasswear prior to extraction. Test tubes were also sonicated in the detergent Decon 90 or washed with concentrated nitric acid (Table 5.10). Samples were also vigorously shaken with methanol, when stoppered, to determine if the stopper was the source. In all of the cases described no significant decrease or increase was recorded in the levels of *N*-butylbenzene sulphonamide observed when analysed using the ion-trap

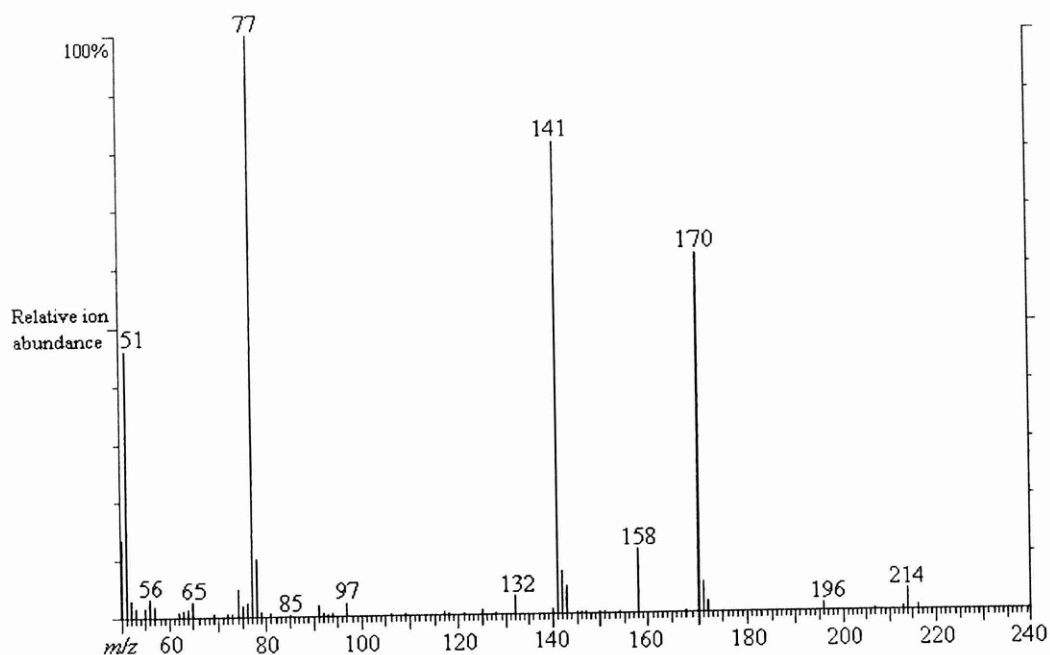


Figure 5.5 The mass spectrum of the sample contaminant which was matched with library spectra and entries in the eight peak index (Table 5.4, below), and was thus identified and *N*-butylbenzene sulphonamide.

Table 5.4 Information from entry in the eight peak index compared with the acquired spectra.

<i>N</i> -butylbenzene sulphonamide (<i>m/z</i> values, Eight Peak Index)	Relative ion abundance (%) (Eight Peak Index)	Relative ion abundance (%) (Sample contaminant)
77	100	100
141	97-74	82
170	99-79	73
51	20-26	45
41	20-10	not scanned
78	16-10	10
158	10	12
171	9-10	5
214	-	5 ([M + H] ⁺ , self-CI)

instrument with retention time approximately that depicted in Figure 5.5 and the key fragments listed in Table 5.4.

From the results discussed above it was considered that the only component that the experiments had in common was the presence of methanol as the extraction solvent. A preliminary GC experiment had suggested that methanol was not the source of contamination but, in case that result had been erroneous, it was deemed necessary to reanalyse the methanol. This time GC/MS was utilized rather than GC alone. A small volume (2 ml) of the solvent was evaporated to dryness and any remaining material dissolved in ethyl acetate and analysed using GC/MS. In this case lactonization was unnecessary as it had already been shown that the contamination was present from the earliest stages of the work-up. The chromatogram from this analysis contained the peak corresponding by retention time and mass spectrometry to *N*-butylbenzene sulphonamide. It was deduced that the original chromatograms for methanol analysis that suggested that the solvent was free of contamination were recorded while the FID detector or the GC system was not operating correctly.

The remaining methanol, which had been used for sample extraction up to this point, was redistilled and used for the standard extraction and this brought about a large reduction in the levels of contamination observed. A small amount of HPLC grade methanol (2 ml) was dried and analysed and the resulting chromatogram was shown to be free of the contaminant. A spiked blood sample was then extracted using HPLC grade methanol. In this case *N*-butylbenzene sulphonamide was not detected. It was shown therefore that the source of contamination was the batch of methanol for these experiments.

In this case it has been possible to identify positively the contaminant as *N*-butylbenzene sulphonamide and for the purpose of this work to eliminate it as a concern to the analysis.

It has not however been possible to pinpoint the overall source of the problem. The results from the experiments outlined above have been presented at a number of meetings. The interest in these findings has been widespread from fellow research workers who have encountered the contaminant from sources similar to those detailed here and a number of groups working on various projects in the field of mass spectrometry have now identified this compound and it appears to be widespread. A major mass spectrometer manufacturer has tuned electrospray mass spectrometric instruments on m/z 214 as it is always present in water: methanol mixtures and analysts in the water industry have reported detection of *N*-butylbenzene sulphonamide water sources from bore water to the purest of treated waters [Elga].

5.4 SEM ANALYSIS OF BLOOD SPOTS.

Experiments were carried out in order to demonstrate any visible changes in the nature of the blood spots due to the various procedures used in their preparation and work-up. These include the effect of the solid carbon dioxide, which is used to keep the samples cold enough to prevent or at least reduce clotting of the fresh blood during transport. The effect of time when Guthrie spots are stored at room temperature and the effect of sonication as an extraction method. Five samples were viewed by scanning electron microscopy and there were approximately six months between the preparation of the older and the fresh samples. Samples were not dried using the technique of critically point drying as the air drying would have already caused the desiccating and disruption of the cells. In the case of fresh blood this method would allow the fixing of the cells without damage.

The results of this analysis have shown that keeping the blood cold during transport (at approximately -40°C with solid CO_2) did not cause any visible damage to the blood cells other than that found in the case (Appendix E1) of the untreated fresh blood, and any

changes can be attributed to the effect of drying causing desiccating of the cells.

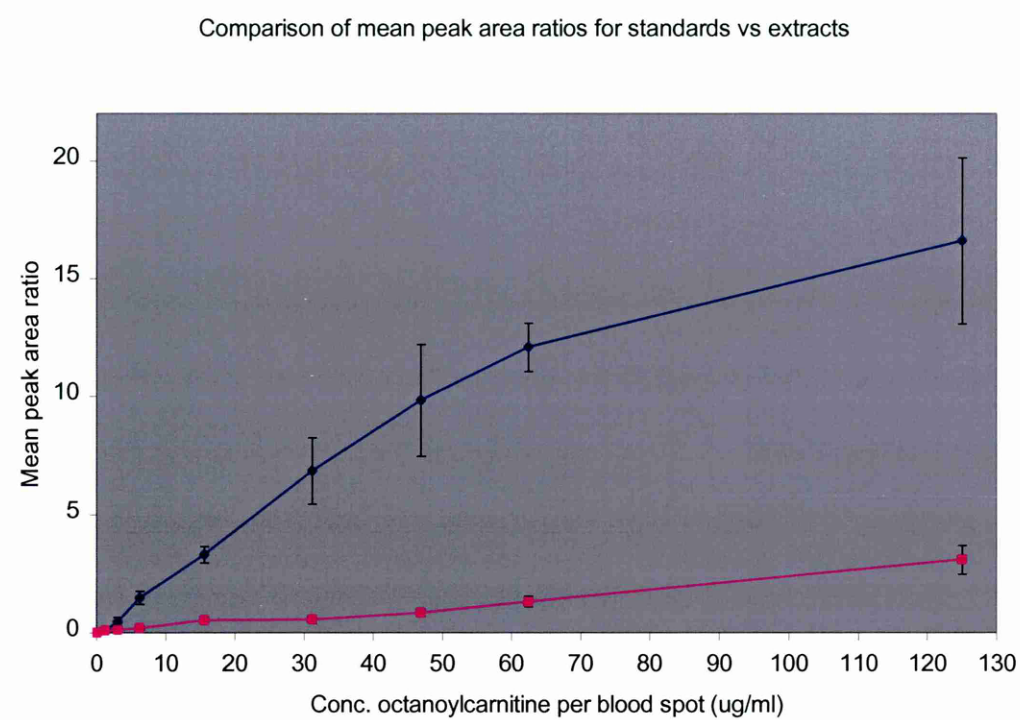
Sonication of the cells was however shown to cause a complete breakdown of the remaining cells and the disruption of the layer of serum-like material which coats the surface of each blood spot (Appendix E2). In the case of non-sonicated samples both red (1-2 μm) (Appendix E4) and white blood cells (20-30 μm) (Appendix E3) were observed. These were identified as blood, and not bacterial cells, as on increase of the probe current over the small area the single membrane of the bacterium would be expected to rupture, whereas the more robust nature of the cell membranes prevents this. SEM prints are shown in Appendix E to illustrate these findings.

Investigation into the effect of time on the condition of the blood spots did not identify any significant changes over the time span involved (six months). This would suggest that the blood spot would be in a condition for analysis without any storage specifications after this time (Appendix E5). Changes to the nature of the acylcarnitines has not been studied, though acylcarnitines have been shown to be stable over long periods of time [4 and Section 5.1 of this thesis].

5.5 QUANTITATIVE EVALUATION OF THE METHOD

It has been shown that a variety of acylcarnitines can be extracted, derivatized and characterized, through the use of capillary gas chromatography coupled with mass spectrometry, from spiked dried blood spots on conventionally available Guthrie cards. Although the analysis of samples of this nature mainly requires a qualitative rather than a quantitative answer, an investigation to estimate the quantitative recovery from the extraction (chloroform/methanol, 2:1) of blood spots relative to that of directly cyclized octanoylcarnitine was undertaken. The latter provides a measure of the optimum yield (80 - 100%) which would ideally be achieved also with blood spots. The direct lactonization

of octanoylcarnitine from dried blood spots (i.e. lactonization directly from Guthrie paper in hot acetonitrile without extraction) was also being assessed. Peak areas were recorded for both octanoylcarnitine and isovalerylcarnitine lactones using the mass chromatogram at m/z 85. The acylcarnitine lactones in this case were identified by retention time, isovalerylcarnitine lactone as internal standard at scan number 505-509 and



octanoylcarnitine lactone at scan number 766-770 and their fragmentation pattern. Concentrations of octanoylcarnitine, peak areas for both acylcarnitine lactones and the peak area ratios are presented graphically in Figure 5.6. Identical levels of isovalerylcarnitine (6.25 μg) were added to each sample.

Data used in the generation of Figure 5.6

Conc. Oct carn/ blood spot	Peak area ratio from standards			Standard deviation	Mean peak area ratio
0	0	0	0	0	0
0.125	0	0.005	0	0.002887	0.001667
1.25	0.195	0.012	0.056	0.09564	0.087567
3.12	0.638	0.298	0.490	0.17066	0.475033
6.25	1.168	1.519	1.733	0.285255	1.473333
15.60	2.900	3.485	3.521	0.348607	3.302
31.20	5.600	8.365	6.562	1.403635	6.8424
46.90	7.249	11.883	10.364	2.362374	9.832033
62.50	11.421	13.254	11.566	1.019008	12.08033
125.00	13.776	20.548	15.435	3.529821	16.56862

Conc. Oct carn/ blood spot	Peak area ratio from extracts			Standard deviation	Mean peak area ratio
0	0.012	0	0	0.0069282	0.004
0.125		0	0	0	0
1.25	0.194	0.080	0	0.0974953	0.091
3.12		0.172	0.070	0.07212489	0.121
6.25	0.122	0.251	0.192	0.06457812	0.188
15.60			0.533		0.533
31.20	0.524	0.736	0.413	0.16411073	0.558
46.90	0.812	0.996	0.715	0.14272701	0.841
62.50	1.140	1.255	1.561	0.21760132	1.319
125.00	2.393	3.309	3.546	0.60891078	3.083

Figures 5.6 A graphical comparison of the peak area ratios for standard octanoylcarnitine solutions versus extracted blood spots and the data sets used to compile it.

A number of samples were prepared in triplicate in order to provide some quantitative insight into the extraction and cyclization of acylcarnitines. There were three differing approaches. The first (a) was to obtain the peak area ratio (octanoylcarnitine/ isovalerylcarnitine) from the cyclization of standard octanoylcarnitine solutions. The second was to generate the same data from extracted blood spots to allow a direct comparison. The third was to directly cyclize the acylcarnitines from blood spots as

described in Experiment 5.11.7. In the first case extraction is not a factor and we are assessing cyclization presuming a 100% extraction efficiency. Over the range (0 – 125 /spot) the graph of peak area ratios the cyclised standards are shown as the first and upper set of data. The second series shown above is the peak area ratio from spiked blood spots, extracted and cyclized, over the same range. A comparison of maximum mean peak area ratios ($a=16.59$, $b=3.08$) would suggest an extraction efficiency of approximately 18% with poor quantitation from blood spots at lower concentrations. In the third case samples were cyclized directly and the recovery was lower (approximately 5%). The graph in Figure 5.6 allows a visual comparison of the first two sets of data. Whilst recovery of about 18% is disappointing for the extracted blood spots, it was decided to try to analyse clinical samples to determine if this recovery is sufficient for diagnosis of disease states.

5.6 APPLICATION TO CLINICAL SAMLES

All clinical samples discussed here originate from patients who had been diagnosed as authentic cases of the disorders in question by means other than the method described here. When dealing with inherited metabolic disorders, samples to test a new method are not easy to obtain but are very necessary to establish the effectiveness of a method prior to its use in the diagnosis of unknown clinical disorders. In this section three examples are presented to demonstrate the applicability of this simple gas chromatographic method to the diagnosis of such diseases in neonatal patients. In normal blood samples (i.e. from people not suffering from any known metabolic disorder) it has not been possible to detect the trace levels of acylcarnitines present. Their concentrations fall below the limit of detection. In all the clinical cases described here therefore a positive result is the detection of any acylcarnitines after extraction of the blood spot and this is considered an elevated response. This situation is clearly not ideal but a significant increase in the extraction yield

would be necessary to record normal acylcarnitine levels, if indeed this detection was possible by GC/MS.

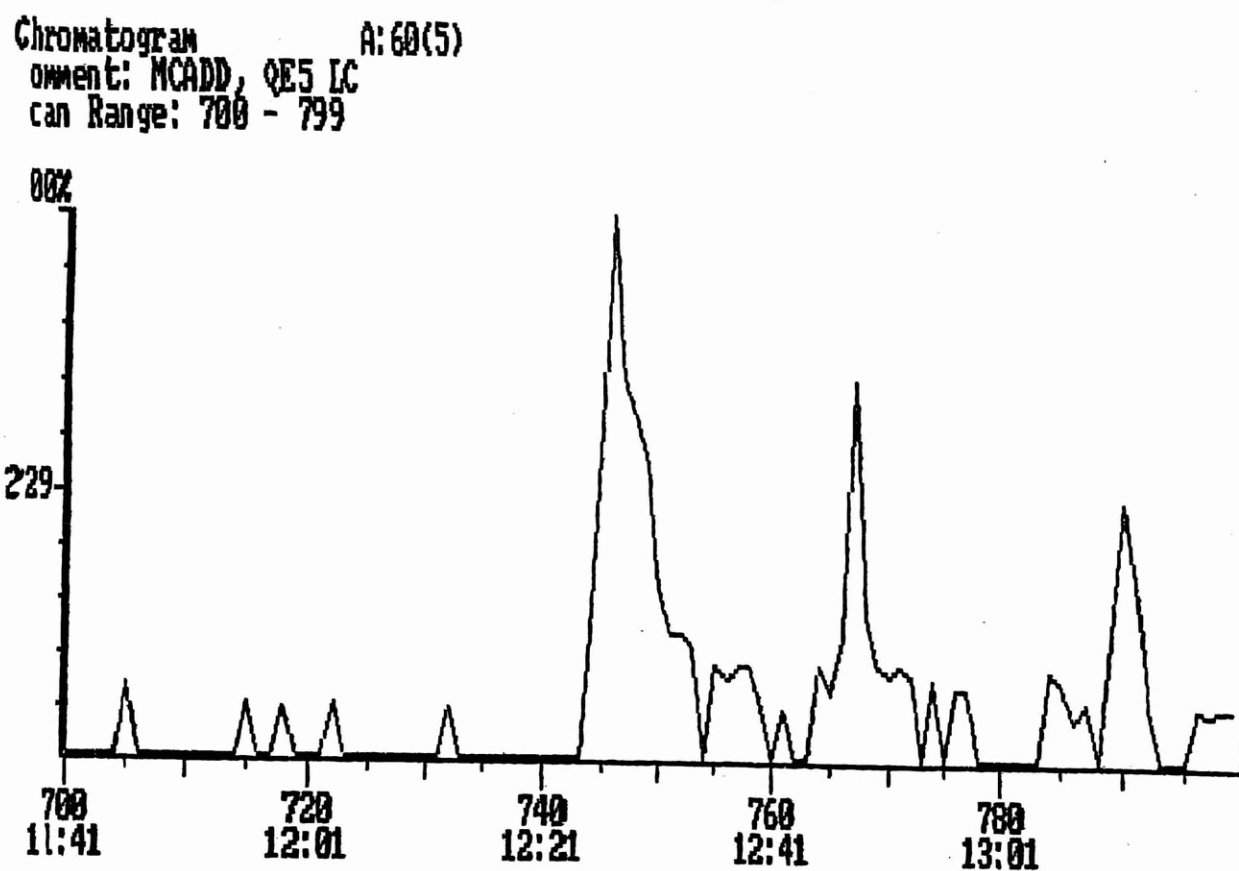
All clinical samples discussed in this section were prepared and analysed in the same manner. Bloods spots, obtained from a number of hospitals, were extracted using the mixed solvent system of chloroform/methanol (2:1) via sonication (2 × 10 min). The solvent aliquots were then dried, and the derivatization reagents added. The cyclized samples were then analysed by GC/CIMS with isobutane on the Finnigan MAT ITD 800A. For the analysis of acylcarnitines using the ion-trap it was considered that chemical ionisation was the most effective mode of ionization. The resulting spectra usually consist of the protonated molecule as the base peak and little fragmentation other than peaks at m/z 85 as in EI. The peak at m/z 125 has not yet been characterized but, due to it is present in most acylcarnitine lactone CI spectra, may be due to a C_3H_4 adduct of the lactone ring ion at m/z 85.

5.7 MEDIUM-CHAIN ACYL-CoA DEHYDROGENASE DEFICIENCY (MCADD).

MCADD is a disorder of one of three mitochondrial matrix acyl-CoA dehydrogenases which carry out the initial dehydrogenation step in the β -oxidation of straight chain fatty acids. This enzyme is responsible for the breakdown of C_6 - C_{10} carbon length fatty acids and if this enzyme were damaged or absent mitochondrial oxidation of endogenous and dietary fatty acids, would be affected [11]. This disorder, medium-chain acyl-CoA dehydrogenase deficiency (MCADD) has been incorrectly diagnosed as Reye's syndrome or SIDS (Sudden infant death syndrome). MCADD is triggered by excessive fasting (> 12 hr) and in some cases is only identified when a sibling is identified as suffering from the disorder. Characteristic of this disorder is a secondary carnitine deficiency and

dicarboxylic aciduria when stressed from fasting. The mechanism for secondary carnitine deficiency in MCADD is unknown but is possibly due to the high levels of excreted octanoylcarnitine [12]. The organic acid profile in this disorder clearly shows the presence of elevated levels of C₆-C₁₀ dicarboxylic acids.

Figure 5.7 (a) Extracted ion chromatogram, m/z 229, and (b) the resulting background subtracted CI mass spectrum from an MCADD sample provided by the Queen Elizabeth Hospital, London.



Average of: 765 to 769 Minus: 778 to 782

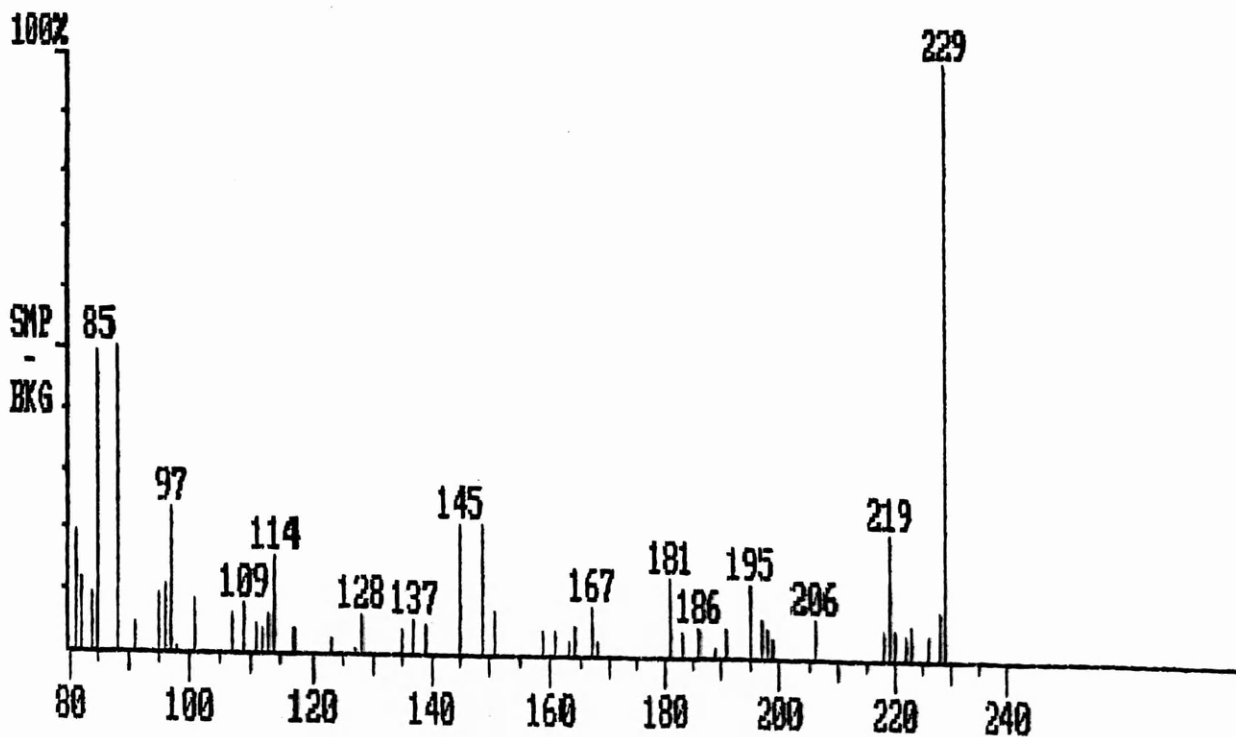
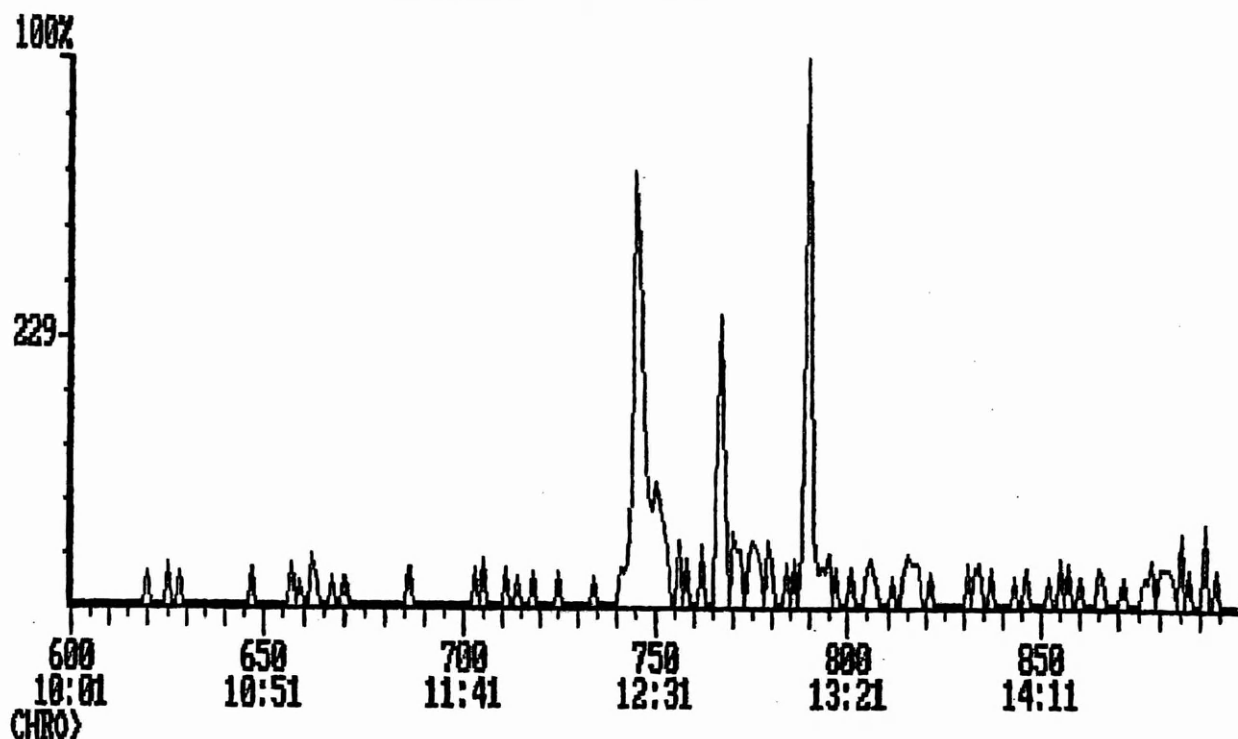


Figure 5.8 (a) Extracted ion chromatogram, m/z 229, and (b) the resulting background subtracted CI mass spectrum from an MCADD sample provided by the Queen Elizabeth Hospital, London.

Chromatogram A:59(11)CI Acquired: May-06-1993 16:11:13
 Comment: MCADD BLOOD SPOT, QE4 SAP
 Scan Range: 600 - 899 Scan: 600 Int = 17754 @ 10:01 100% = 517



Average of: 765 to 769 Minus: 759 to 763 100% = 102

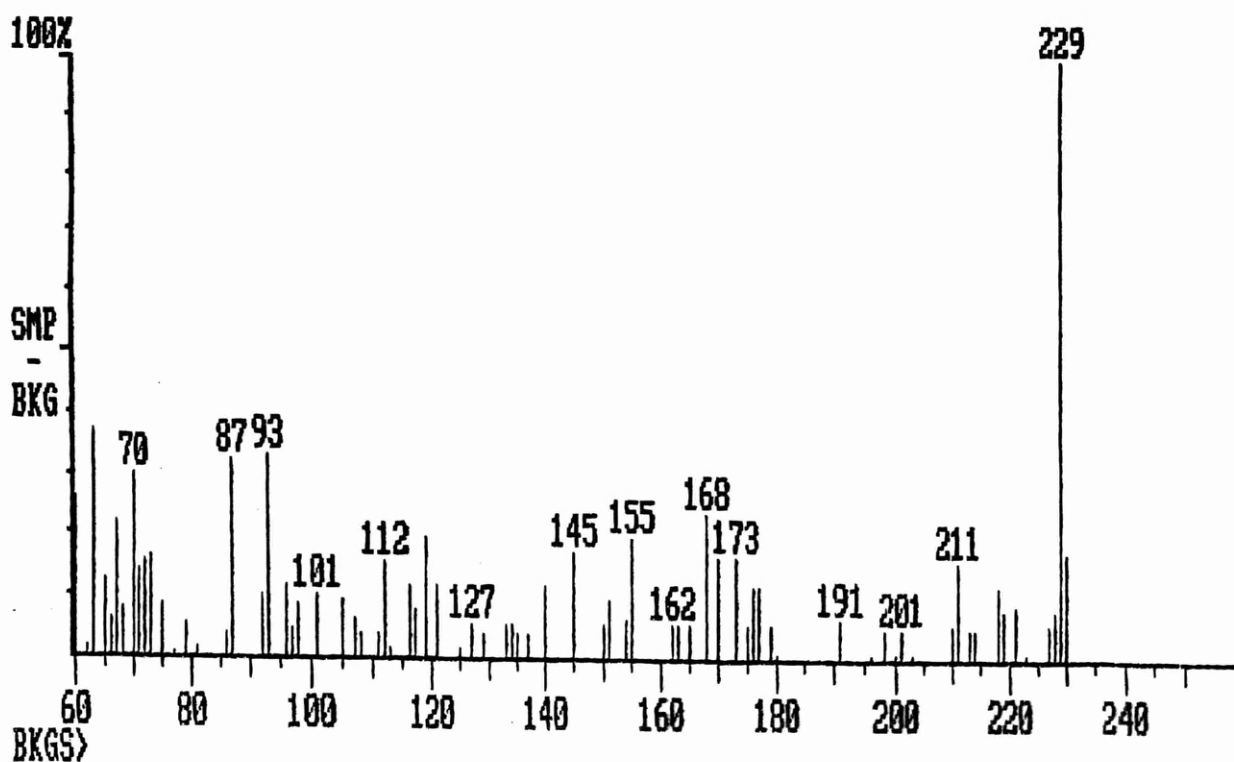
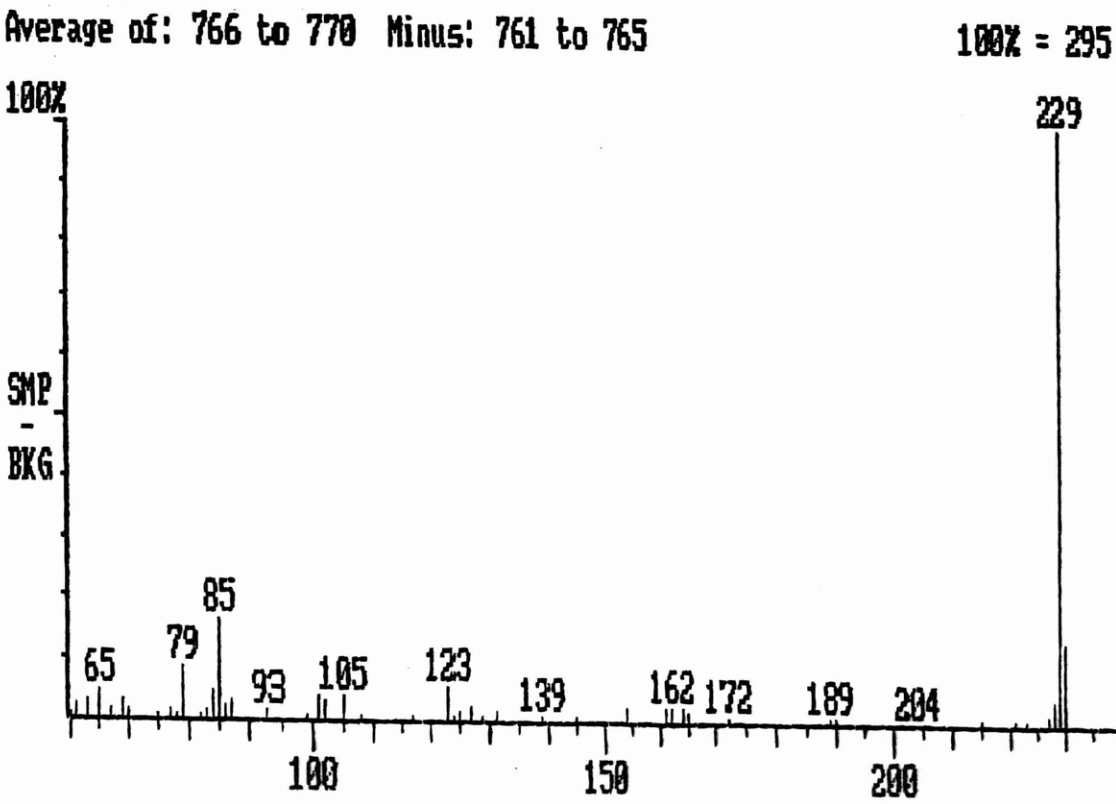
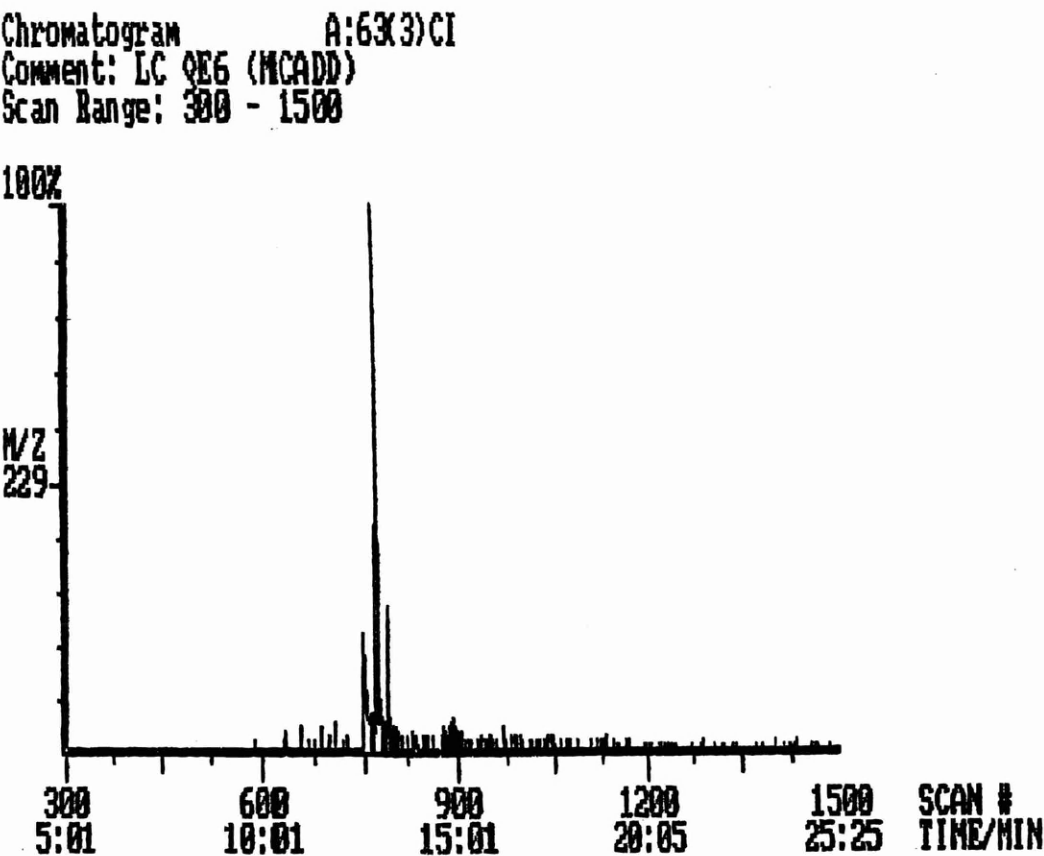


Figure 5.9 (a) Extracted ion chromatogram, m/z 229, and (b) the resulting background subtracted CI mass spectrum from an MCADD sample provided by the Queen Elizabeth Hospital, London.



In acute patients elevated levels of urea and ammonia suggest that proteolysis is accelerated but is unable to meet the demand for substrate needed for gluconeogenesis. Accelerated tissue catabolism and ATP depletion may account for the increase in uric acid levels found at the time of illness [13].

Chromatograms from the analysis of three MCADD clinical blood spot samples are shown in Figures 5.7– 5.9, pages 146-148. GC/MS analysis was carried out in the chemical ionisation mode using isobutane as described in Section 2.3 with particular emphasis on the 700-800 scan number region. Extracted ion chromatograms were obtained for m/z 229 ($[M+H]^+$) and a spectrum of the peak in the specific region of scan numbers 765-770, that characterised for the octanoylcarnitine lactone, is shown for each of the blood spot samples.

These samples clearly show that, although at very low levels, the octanoylcarnitine lactone was clearly discernible in the area of interest and the spectra generated from the peaks in this region have m/z 229 as their base peak. It has therefore been clearly shown here that using octanoylcarnitine lactone MCADD can be diagnosed from blood spots.

5.8 PROPIONYL ACIDEMIA.

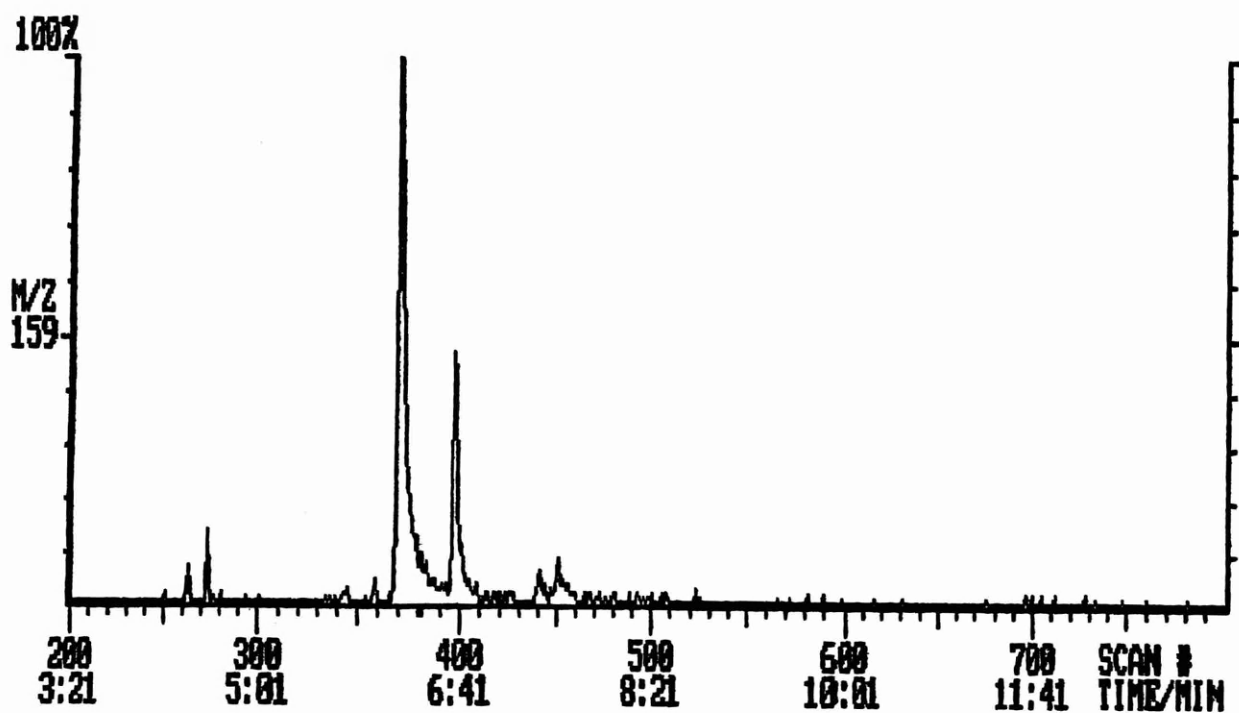
Propanoic acid and other volatile fatty acids are found in high concentrations in ruminants. Non-ruminants however have a low blood concentration of these acids. Other sources of propanoate include the β -oxidation of odd-chain number long-chain fatty acids, the catabolism of amino acids (isoleucine, valine, threonine and methionine) [14] and from thymine. It is the only fatty acid, which in the liver and the kidneys can be converted to glucose [15]. Propanoate is activated to its CoA ester inside the mitochondrial matrix [16] and is further conjugated with carnitine to facilitate reversible transport across the

mitochondrial membrane. The enzyme carnitine acetyl transferase (CAT) is responsible for this conjugation. If the transport of propanoyl-CoA from the membrane is inhibited this produces a toxic effect and the excretion of accumulated CoA esters as their carnitine esters as discussed earlier. Propanoyl-CoA also has the effect of allosterically hindering the action of succinate-CoA ligase, which is responsible for the conversion of succinyl-CoA to succinate [17]. This can cause a decrease in the production of GTP (guanine triphosphate) at the substrate level thus reducing ATP-dependent mitochondrial fatty-acid oxidation [18]. Further to conjugation with carnitine, propanoyl-CoA undergoes a carboxylation, mediated by propanoyl-CoA carboxylase [19], in the mitochondrial matrix to produce D-methylmalonyl-CoA. This is then racemized to the L- isomer, via methylmalonyl racemase, which in the presence of methylmalonyl-CoA mutase yields succinyl-CoA for entry into the energy-generating citric acid cycle [20]. It is abnormal or low activity of the biotin dependent enzyme propanoyl-CoA carboxylase, which gives rise to the diagnosis of propionyl acidemia [19]. The incidence of propionyl acidemia has been reported as 1 in 350,000 birth in one screening program [21].

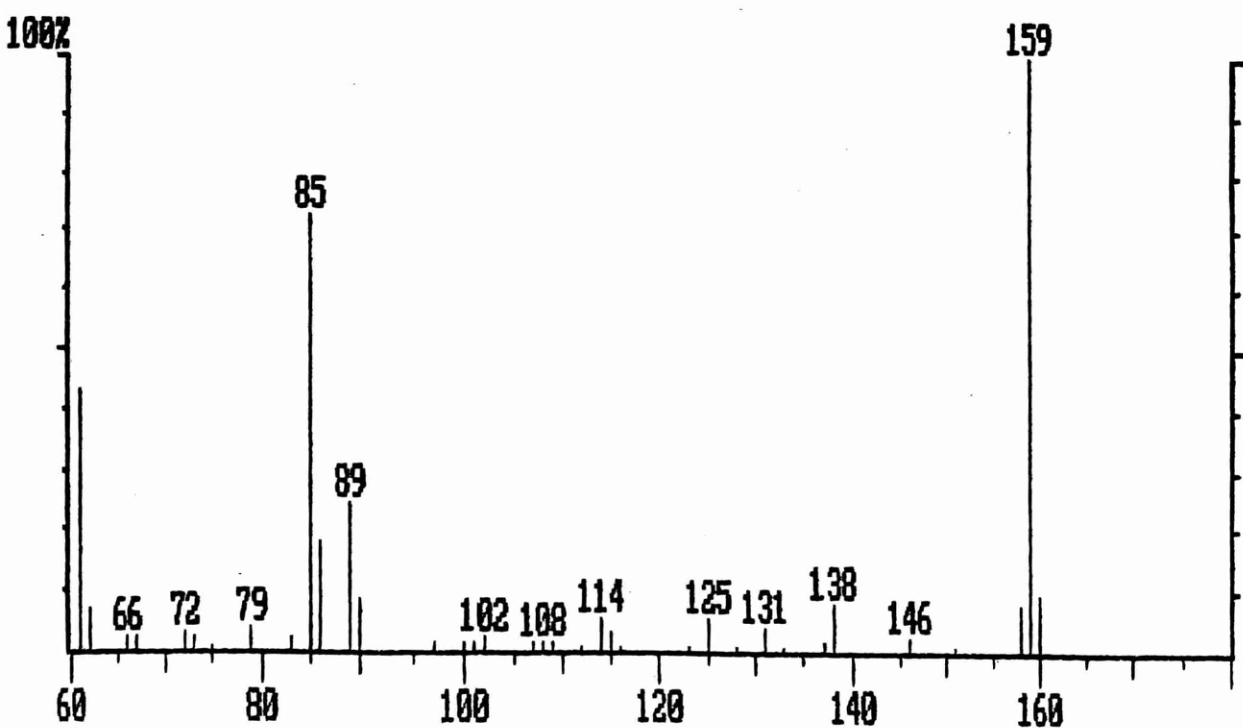
In the disease propionyl acidemia the major urinary metabolite is reported as 2-methylcitrate [20, 22, 23] with the disease characterized by severe metabolic decompensation with metabolic acidosis and hyperammonaemia. Propionyl acidemia is one of the most severe acidemias, which occur in infancy, with acidotic attacks being fatal in up to 40% of patients [24]. Treatment with d,l-carnitine in the oral form has been shown to increase the free carnitine level in plasma to near normal levels and an increase in muscle tone in patients was observed without any adverse effects [22]. Analysis by alkaline hydrolysis paper/chromatography has demonstrated that approximately 90% of the content of acylcarnitines present in a urine sample was propanoylcarnitine coupled with a marked decrease in the levels of methylcitrate [25].

Figure 5.10 (a) Extracted ion chromatogram, m/z 159, and (b) the resulting background subtracted CI mass spectrum from a Propionic Acidemia sample provided by the Temple Street Hospital, Dublin.

Chromatogram A:63(9)CI
Comment: PA1 TS (PROPRIONIC ACIDEMIA)
Scan Range: 200 - 799



Average of: 395 to 399 Minus: 410 to 414



Propionyl-L-carnitine has also been studied in relation to cardiovascular drug therapy with its effect being proposed as biphasic. Penetration of the cytosolic endothelial cells and cardiomyocytes by propanoylcarnitine can improve energy supply. This occurs through the conversion of propanoate to succinate to oxaloacetate [26] which can lower a high acyl-CoA/CoA ratio which can occur in the case of depletion of mitochondrial dicarboxylic acids, slowing down the citric acid cycle and thus reducing ATP generation. It may also give some protection to plasma membranes during ischemia and other associated acidosis [27]

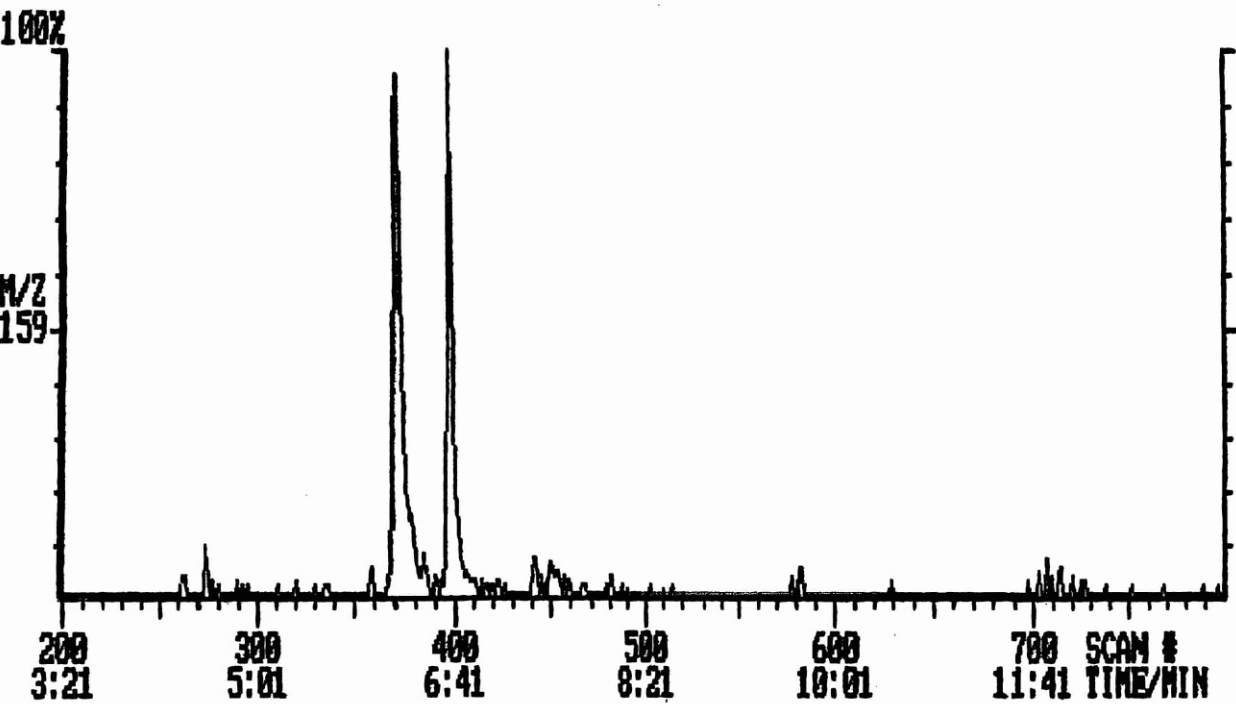
Accumulation of propanoylcarnitine was directly identified first by Millington *et al* using FAB-MS/MS in 1984 [28].

In the work presented here, propanoylcarnitine lactone readily detected in a blood spot from a neonatal patient who had been diagnosed as suffering from propionic acidemia.

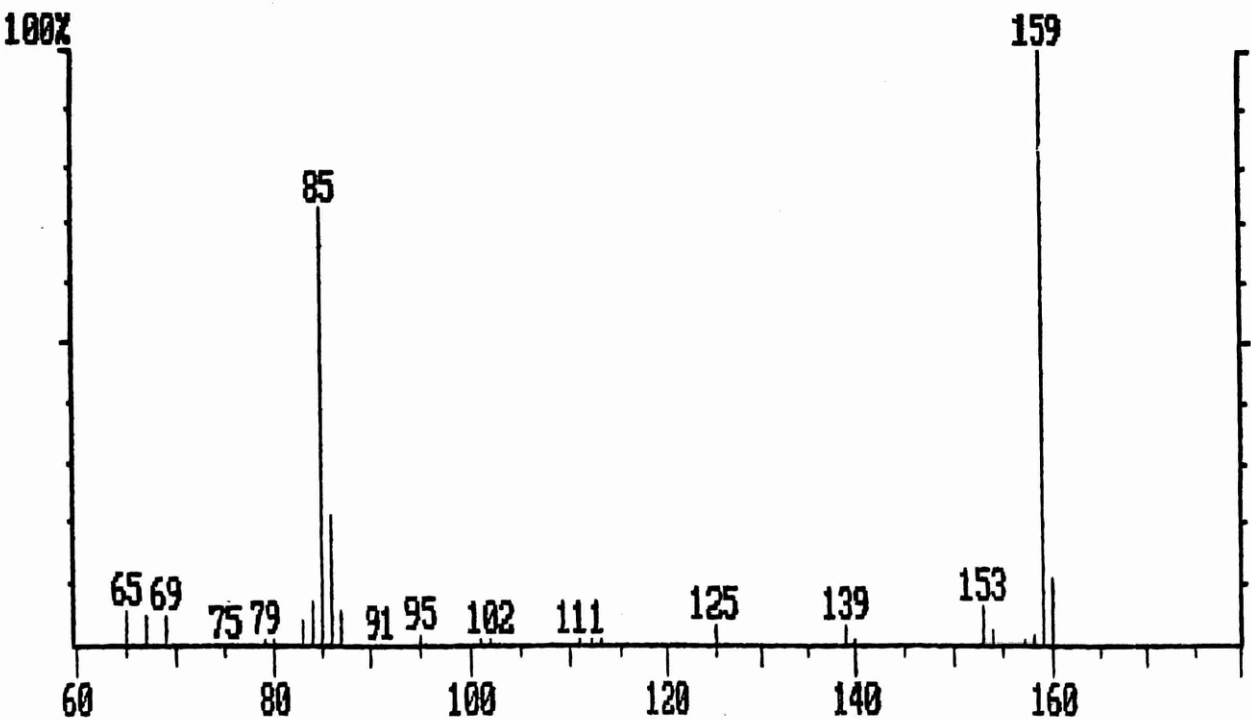
The data presented in Figure 5.10 show propanoylcarnitine lactone detected at scan number 400 in the CI mass chromatogram of m/z 159 (a), the protonated molecule of propanoylcarnitine lactone. The only other peak present in the mass chromatogram is attributed to nonanoic acid. Organic acids of varying chain length have been detected both in the cases of normal and clinical dried blood spots on analysis by gas chromatography. The mass spectrum of propanoylcarnitine from (a) is shown below (b). The base peak in this spectrum is the protonated molecular ion, m/z 159, diagnostic fragments at m/z 85 (73%) and 125 (8%) also present. The peak assigned in this case to propanoylcarnitine matches, both in chromatographic retention time and the mass spectrum generated, that of commercially obtained standard material.

Figure 5.11 (a) Extracted ion chromatogram, m/z 159, and (b) the resulting background subtracted CI mass spectrum from a Methylmalonic Aciduria sample provided by the Temple Street Hospital, Dublin.

Chromatogram A:63(10)CI
Comment: MM1 IS (METHYLMALONIC ACIDURIA)
Scan Range: 200 - 799



Average of: 395 to 399 Minus: 387 to 391



5.9 METHYLMALONIC ACIDURIA.

Methylmalonic acidemia has been shown to be caused by the absence/deficiency of methylmalonyl-CoA mutase or by abnormalities of intra-mitochondrial cobalamin metabolism [29] with its incidence reported as 1 in 48,000 [30]. Its diagnosis can be made through clinical manifestations such as attacks of ketoacidosis and hyperammonemia. Attacks can occur in situations such as heavy protein feeding [31] or infections [32, 33, and 34].

In a blood spot, like the example opposite, propanoylcarnitine lactone was readily detected from a neonatal patient who had been diagnosed as suffering from methylmalonic acidemia. The data presented in Figure 5.11 again show propanoylcarnitine lactone detected at scan number 400 in the CI mass chromatogram of m/z 159 (a), the protonated molecule of propanoylcarnitine lactone. The only other peak present in the mass chromatogram is attributed again to nonanoic acid. The mass spectrum of propanoylcarnitine from (a) is shown below (b). The base peak in this spectrum is the protonated molecule, m/z 159, diagnostic fragments at m/z 85 (73%) and 125 (5%) were also present. A second acylcarnitine, which might be extracted from the blood of patients with methylmalonic acidemia, is methylmalonylcarnitine, which was not detected in this sample either in the total ion chromatogram or on selective ion monitoring of potential key fragments. Methylmalonylcarnitine may not be extracted efficiently because of its different polarity and solubility or, as shown in Chapter 4, it may not be cyclised efficiently under standard lactonization conditions.

5.10 CONCLUSION

In conclusion a method is evolving which allows the analysis of dried blood spots on Guthrie cards. The application of this method to a range of acylcarnitines in spiked blood spots has also been described. A technique has been presented therefore which meets the aim of providing a relatively simple analytical technique, with the potential for use within existing hospital laboratories, using existing or relatively inexpensive instrumentation. The application of this technique to a number of clinical samples has also been illustrated with GC/CIMS providing easily interpreted chromatograms and mass spectra. The potentials for developing this method further are easy to see: a significant increase in the extraction yield is the major requirement. In this way one would hope to monitor trace as well as major acylcarnitine components, and normal as well as elevated levels thus providing a more comprehensive diagnostic service. Considering the complexity of the blood matrix even the total ion chromatograms have relatively low noise levels and few other significant peaks.

5.11 EXPERIMENTAL

5.11.1 REAGENTS AND MATERIALS.

Guthrie cards were donated by Milton Keynes Hospital and the Royal Army Medical College, London and were produced by Whatman (Maidstone, UK). Acylcarnitines were synthesised using previously reported methods [1, 9] or purchased from Sigma (St. Louis, UK) as was the hexan-1-ol. *N,N*-diisopropylethylamine, hexane, pentan-2-ol and butan-1-ol were purchased from Aldrich (Gillingham, UK). Methanol, chloroform, acetone (Analar) and Nitric acid were acquired from BDH Merck (Poole, UK) while HPLC grade methanol was obtained from Rathburn (Walkerburn, Scotland). Reacti-vials (1 ml) were purchased from Pierce (Chester, UK), acrodisc filters (0.2 µm) from Gelman (Northampton, UK), glass syringes from Weber Scientific (Teddington, UK) and stoppered Pyrex test-tubes (10 ml) from BDH Merck. Diethyl ether was obtained from Rhone-Poulenc (Manchester, UK). Glycerol used as the FAB matrix solvent was purchased from BDH Merck. For SEM studies Cambridge stubs were obtained from Bio-Rad /Fisons (E. Sussex, UK) and these were coated by an EM SC 500 sputter coater with a gold target also available from Bio-Rad. Freeze-drying was carried out using a Genevac (Ipswich, UK) SF50 spin freezer, freeze dryer and centrifugal evaporator with a CVP 100 MK4 vapour vacuum pump.

5.11.2 PREPARATION OF BLOOD SPOTS.

Whole blood (10 ml) obtained from a healthy male volunteer was stored in a flask of solid carbon dioxide for 5 - 10 min after donation. This was then mixed for 10 minutes at room temperature to ensure homogenous defrosting. The 10 ml sample then divided into 0.5 ml aliquots.

Each aliquot was then spiked with an appropriate volume of octanoylcarnitine solution as detailed in Table 5.1. Sample aliquots were then vigorously shaken for ten minutes and each was used to produce four blood spots onto Guthrie paper. These papers were then stored at room temperature and were left for at least 2-3 days prior to analysis in order to ensure the drying of the blood spot and to mimic the preparation conditions for later analysis of clinical samples.

5.11.3 DEVELOPMENT OF EXTRACTION PROCEDURE

(i) PRELIMINARY SOLVENT EVALUATION

Blood spots were prepared as detailed above (5.11.2). An area 6 mm in diameter was punched for the blood spot and this was further cut into a number of segments that were placed in a stoppered test tube.

Octanoylcarnitine was extracted by vigorously shaking the dissected Guthrie spot twice in methanol (2 x 1 ml). The combined extracts were then freeze dried and the residue dissolved in 200 µl of acetonitrile. FAB/MS was carried out on the residue to determine if the analyte was successfully extracted.

The standard lactonization procedure was then carried out on the extracted acylcarnitine to produce the volatile octanoylcarnitine lactone. GC/MS analysis of the samples was then carried out to confirm the presence of octanoylcarnitine lactone.

(ii) EVALUATION OF PHYSICAL EXTRACTION

A card of four blood spots was prepared as detailed in the previous experiment (5.11.3 (i)). Three areas 6 mm in diameter were punched from the card and dissected into smaller segments. These segments were placed in test tubes and 1ml of methanol was added to each tube. The samples were then treated in differing manners as follows;

- (i) Sample was shaken for 10 min
- (ii) Sample was sonicated for 10 min
- (iii) Sample was vortex mixed for 10 min.

Each of these processes was repeated with a further 1 ml of methanol. The combined extracts were freeze dried and the resulting residue dissolved in 200 μ l of acetonitrile.

The standard lactonization procedure was then carried out on the extracted acylcarnitine to produce the volatile octanoylcarnitine lactone. Followed by GC/MS analysis.

(iv) DURATION OF PHYSICAL EXTRACTION

Dried, spiked blood spots were prepared and extracted in the normal manner.

Isovalerylcarnitine was added to the extraction solvent prior to sonication as an external standard. The sonication time of the samples was varied from 2 - 30 min for each millilitre of solvent (as Table 5.2)

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5.11.4 SPIKING EFFICIENCY

A standard section (6 mm diameter) was excised from a spiked Guthrie blood spot and the acylcarnitines were extracted and cyclized. The remaining area of blood spot was then treated in the same manner. The calculated areas of these two portions of blood spot were approximately the same.

A blank Guthrie paper was spiked with the same concentration of octanoylcarnitine as above. Inner and outer areas of Guthrie spot were the prepared as for the blood spot.

All four segments of paper were extracted in the same manner and the resulting residues cyclized. The samples were then analysed by GC/MS (Table 5.8).

5.11.5 ELECTROSPRAY ANALYSIS OF EXTRACTION RESIDUE

Guthrie spots spiked with octanoylcarnitine solution (as Table 5.1) extracted with methanol and the resulting residues containing the acylcarnitine and any other co-extracted materials were analysed by electrospray ionization (ESI/MS); see Chapter 6 for further details of this analytical technique.

The extraction residue was reconstituted in 100 µl of a chloroform/methanol mixture and analysis was by loop injection. Varied injection volumes were used to determine if the residue contained acylcarnitine at levels suitable for analysis or other co-extracted material.

5.11.6 EXTRACTION OPTIMIZATION

A series of experiments was designed to determine if an alternative solvent, or mixture of solvents, could be found which facilitated the extraction of octanoylcarnitine, without that of proteins and/or other compounds, which may inhibit cyclization

(i) SINGLE SOLVENT SYSTEMS

A number of single solvent systems were used in an attempt to improve the yield of lactones for analysis (Table 5.3). Pentan-2-ol, 1-butanol and 2-hexanol were used in place of methanol. Sample analysis was by GC/EIMS using an ion-trap

TABLE 5.5 – Evaluation of individual extraction solvents

	Solvent	Mean peak area octanoylcarnitine lactone (N=2)
A	Pentan-2-ol	16998
B	Hexan-2-ol	22114
C	Butan-1-ol (HPLC Grade)	14772

(ii) MIXED SOLVENT SYSTEMS

Solvent mixtures were evaluated to determine if one solvent could facilitate protein immobilization while extraction was the role of the second.

Blood spots were prepared with 6.25 µg of octanoylcarnitine/blood spot. Acetone and ethanol were combined with varying volumes of methanol for extraction (Table 5.4). The method of extraction was otherwise unchanged from Experiment 5.11.3.

A comparison was then carried out between this preferred acetone/methanol system and a solvent system of chloroform/methanol in a 2:1 ratio (as used for the analysis of acylcarnitines from urine [8]).

TABLE 5.6 - Evaluation of mixed extraction solvents (Table 5.3, page 133 shows results from this experiment).

	Methanol (%)	Acetone (%)	Ethanol (%)
A	90	10	0
B	75	25	0
C	65	35	0
D	50	50	0
E	0	100	0
F	0	0	100

5.11.7 MODIFICATION OF EXTRACTION TIME

A series of experiments were designed to determine (i) if the use of microwaves would allow the cyclization time to be cut to minutes or even seconds (ii) if direct cyclization of samples was viable.

(i) USE OF MICROWAVES

Spiked blood spots were prepared (5.11.2) and extracted (5.11.3) in the normal manner with methanol. One set of blood spots spiked with a known concentration of octanoylcarnitine were cyclized in the normal manner as a control sample.

Samples of identical octanoylcarnitine concentration were prepared and these were subjected to a variety of microwave intensities and durations (Table 5.7). Samples were then analysed by GC/MS to compare the levels of octanoylcarnitine lactone generated.

Table 5.7 – Microwave intensities and duration

Sample	Microwave Settings	Capping	Time/ mins	Mean peak area octanoylcarnitne lactone (N=3)
1	Low/water sink	Loose	0.5	ND
2	Low	Loose	0.5	ND
3	Medium	Tight	0.5	ND
4	Low	Tight	1.0	7364
5	Medium	Tight	1.0	9451
6	Low	Tight	1.0	8521
7	Medium	Tight	1.0	13213
8	Low	Tight	2.0	15135
9	Medium	Tight	1.5	19111
10	Medium	Tight	2.0	35131

(ii) DIRECT EXTRACTION AND CYCLIZATION OF BLOOD SPOTS.

A spiked blood spot (6 mm) was added to a Reacti-vial containing 200µl acetonitrile and 80 µl DPEA solution, this mixture was then cyclized in the normal manner (125°C/ 35 min). The Guthrie paper was then removed and the solvent dried under a stream of nitrogen. The resulting residue was then dissolved in 50 µl of ethyl acetate and analysed by GC/MS. The resulting peaks were compared with the control sample from (5.11.7 (i)).

5.11.8 ANALYTE RECOVERY

An investigation was undertaken to estimate the quantitative recovery from the extraction/cyclization of octanoylcarnitine blood spots and to compare this to that of directly cyclized octanoylcarnitine. Isovalerylcarnitine (6.25 µg) has again been used as an external standard being added to the extraction solvent in the case of the extracted/cyclized samples and to the Reacti-vial for cyclization in the case of the directly cyclized samples.

Peak areas were recorded for both octanoylcarnitine and isovalerylcarnitine lactones using the mass chromatogram at m/z 85. The acylcarnitine lactones in this case were identified by retention time, isovalerylcarnitine lactone as internal standard at scan number 505-509 and octanoylcarnitine lactone at scan number 766-770 and their fragmentation pattern

The efficiency of the extraction stage was investigated by carrying out the experiments described in Table 5.8.

TABLE 5.8 – Experiments designed to evaluate spiking and extraction efficiency

Sample	Work-up	Mean peak area octanoylcarnitine lactone (N=4)
1	Blood (conc. 15.6ug/ml), methanol/acetone extract , and cyclization.	3588
2	Octanoylcarnitine (6.3 ml of 500 mg/ml) soln., cyclization.	36252
3	Pre-prepared spot + octanoylcarnitine as 2, MeOH/Acetone, cyclization.	3032
4	Excised paper spot + octanoylcarnitine as 2, MeOH/Acetone, cyclization.	1426
5	Remaining Guthrie paper from 4 (outside).	6248
6	Remaining blood spot from 1 (outside).	4520

5.11.9 EXTRACTION OF ACYLCARNITINE MIXTURES.

Solutions of a number of acylcarnitines were prepared.; butanoyl- (synthesized), hexanoyl-, octanoyl- and dodecanoylcarnitine all at 5 mg/ml. A number of blood spots were then prepared (as detailed for the five highest concentrations in Table 5.1). These spots contained all four acylcarnitines.

Blood spots were prepared in the following manner. For example at the highest concentration, 200ul aliquots of each 5 mg/ml solution were combined in a sample vessel

and the mixture freeze dried. The resulting acylcarnitine residue was dissolved in 0.5 ml of blood and mixed well was used to spike four of Guthrie blood spots giving 125 ug of each acylcarnitine per blood spot. For aliquot volumes at other concentrations see Table 5.1).

A second set of blood spots containing an acylcarnitine mixture was prepared using butanoyl-, isovaleryl-, octanoyl- and dodecanoyl-carnitines. The blood spots were prepared in the same manner as detailed above.

5.11.10 APPLICATION TO CLINICAL SAMPLES.

All clinical samples discussed originate from patients who had been diagnosed as authentic cases of the disorders in question by means other than the method described here.

Bloods spots, obtained from a number of hospitals, were extracted using the mixed solvent system of chloroform/methanol (2:1) via sonication (2 x 10 min). The solvent aliquots were then dried, and the residue cyclized.

Samples were analysed by GC/CIMS with isobutane on the Finnigan MAT ITD 800A under conditions given in Section 2.3 (ii).

5.11.11 SAMPLE CONTAMINATION - AN INVESTIGATION

(i) SOLVENT INVESTIGATION.

A 2 ml sample of methanol taken through the extraction procedure for acylcarnitines. The residue after freeze drying was lactonized. Glassware and solvent were those which had been used in previous experiments.

This procedure was repeated using HPLC grade methanol.

(ii) TESTING OF GUTHRIE PAPER

Non-spiked Guthrie paper was initially extracted with 2 ml methanol and the resulting residue lactonized in the usual manner.

Solvent pre-washes of the paper were also carried out with the solvents listed below (Table 5.9) any reduction in levels of contamination. All samples were prepared in the glassware that had previously been used and cleaned as normal.

TABLE 5.9 – Solvents for Guthrie paper pre-wash

Solvent Volume	Solvent
2 X 1 ml	Hexane
2 X 1 ml	Diethyl ether
2 X 1 ml	Ethyl acetate

(iii) ENVIRONMENTAL CONTAMINATION EVALUATION

Guthrie spots were excised from cards without contact by hand, through the use of a hole punch or scissors. Alternatively the spots were heavily handled to ensure the occurrence of any personal contamination that might normally occur. The work-up and analysis were carried out in the normal way.

(iv) DETERGENT CONTAMINATION

The glassware used in all the experiments involving blood spots was identical and consisted of 10 ml Pyrex stoppered test tubes and sample tubes (1 ml). Sample tubes were discarded after use.

TABLE 5.10 – Glassware – pre-extraction procedure

Sample	Pre-extraction procedure
1	Untreated
2	Methanol (soak 10 ml/10 min)
3	Decon 90 (sonication x 20 min.)
4	Nitric acid (soak 10 ml/30 min)

Glassware was treated as detailed in Table 5.10 prior to extraction and analysis of resulting samples by GC to ensure that the contamination source was not the test tube stoppers. Test tubes were either left standing, after addition of each millilitre of solvent (2 x 1 ml), to prevent contact with the stopper or the solvent aliquots were vigorously shaken to ensure maximum contact with the tube stopper. The normal work-up and analysis followed both procedures.

5.11.12 SCANNING ELECTRON MICROSCOPY (SEM) OF BLOOD SPOTS.

(i) SAMPLE PREPARATION.

Spiked Guthrie spot samples were prepared in the usual manner (Experiment 5.11.2).

Sample A (Table 5.11) fresh blood was spotted onto a Guthrie card (without spiking). Samples B and C spiked blood spots were prepared as normal and extracted using methanol (2 x 1 ml). This extraction was brought about through vigorous shaking. Samples D and E were treated as the previous two samples but extraction was by sonication. The spots, when dry, were analysed by SEM.

(ii) SCANNING ELECTRON MICROSCOPE (SEM) ANALYSIS.

Each excised blood spot was halved and then quartered with the opposite sides of two quarters mounted upward on a standard Cambridge stub with carbon tab. This was then coated with an even layer of gold, using a sputter coated with a gold target (20 mv for 60 seconds). The stub was then transferred to the SEM (LICA 360 Stereo scan SEM) and examined at an accelerating voltage of 12 KeV, which is considered a low voltage.

Samples C and D were prepared six months prior to SEM analysis.

TABLE 5.11 – Sample preparation for SEM

SAMPLE	SAMPLE PREPARATION METHOD
A	Fresh blood/ untreated.
B	Solid CO ₂ (10 min), octanoylcarnitine spike
C	As B.
D	As B, and spot sonicated in methanol (2 x 10 min).
E	As D.

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CHAPTER 6.

ANALYSIS OF ACYLCARNITINES BY ELECTROSPRAY MASS SPECTROMETRY.

6.1 INTRODUCTION.

Methods available for the analysis of carnitine and acylcarnitines have been discussed in Chapter 1 but in the area of analytical science the development of new instrumentation is on going and this chapter contains a discussion of work carried out in collaboration with VG BioTech (Fisons Instruments, Altrincham, UK.) toward the development of a novel method for the analysis of acylcarnitines. Most approaches to the analysis of acylcarnitines involve the derivatization of the zwitterionic molecules to enhance some aspect, be it the volatility of the acylcarnitines for GC analysis or their chromophoric properties as is often the case for HPLC. Ideally though analysis would be carried out on underivatized acylcarnitines and the method of electrospray mass spectrometry [ESI-MS] has now been investigated towards this end [1] with its routine nature offering an affordable and attractive method.

Although electrospray is a relative newcomer to the analytical field the theory on which the technique is based originated in the early part of this century, with the work of Zeleny [2]. This involved the concept that fine sprays of charged droplets can give rise to a strong electrostatic field. It was demonstrated that if an analyte solution was passed, via a small tube, into a nitrogen atmosphere and at atmospheric pressure this spray could be achieved. This work was later reborn and investigated in the context of mass spectrometry [3, 4]

Dole *et al.* proposed a model for electrospray, which is known as the Charged Residue Model [3,4]. In this case it was postulated that with desolvation of the analyte droplets there was an increase in the surface-charge density. When this loss of solvent caused the surface-charge density to reach its critical point (Rayleigh Limit, when the Coulombic repulsion and surface tension are comparable) [5] the instability created causes eruption

(Coulombic Explosions) of the parent droplet to produce offspring. This continues for a number of generations until one is left with a droplet so small that it will contain only one analyte molecule. This single molecule, due to the retention of the droplet charge during desolvation, becomes an ion [6].

Iribarne and Thomson [7,8,9] proposed a second model with similar origins in 1976. This Atmospheric Pressure Ion Evaporation Model argues that in the series of coulombic explosions the droplet offspring that are produced eventually reach a point where the radii are so reduced and the charge density so elevated that an electrostatic field is produced at the droplets' surface. This field then has sufficient intensity to raise the analyte ions into a surrounding ambient gas or vapour without requiring the one analyte per droplet stage of Dole. This method of sample nebulization is often referred to as aerospray (AS) due the pneumatic generation of droplets.

This work was not however immediately utilized. It was Vestal *et al.* (1980) [10,11] who evolved the technique as thermospray ionization. In this case a sample was passed through a narrow tube with heated walls to facilitate vaporization of the carrier solvent.

Nebulization is then brought about by the acceleration and expansion of the vapour, which emerges as a jet at the end of the tube. Though the link with the earlier work of Dole, Iribarne and Thomson was not initially made, thermospray ion formation was later attributed to the formation of charged droplets during nebulization. It has also been proposed that the thermospray ions are formed in the process of collisions between solute and ions of the volatile buffer solvent of liquid chromatography (LC) in the case of LC/thermospray mass spectrometry [6].

Electrospray involves the addition of the analyte to a carrier solution (often water/acetonitrile) through sample injection. A high voltage at the injection point allows

dispersion of the emerging liquid into a fine spray. A flow of high velocity gas around the needle can aid in the stabilization of the spray and can allow the use of increased flow rates. Under these conditions the spray drops are desolvated in a stream of dry gas until all the carrier solution is removed releasing the compounds of interest into the atmospheric pressure source for analysis. Droplets produced in electrospray are formed by electrostatic forces, as in the case of Dole and can be expected to have higher mass-to-charge ratios than the other techniques described. Higher analytical sensitivity can thus be produced [6].

Although the mechanism of droplet formation and analysis utilized in the techniques described is as yet unconfirmed the most likely explanation is thought to be that of Iribarne and Thomson [6]. There are those however who remain faithful to the Dole charged residue model [12]. In the carrier solution acylcarnitines will exist as protonated molecules (cations). These cationic molecules being singly charged will give one significant peak in the positive-ion mode of electrospray as they do not possess a strong basic site within their structure where further protonation may have otherwise occurred.

Early coupling of electrospray to mass spectrometers [13, 14] opened the way for an explosion in the growth of many varied of mass spectrometric application [15]. The most notable application being the analysis of large biopolymers through efficient ionization and its multiple-charging properties together with coupling with a number of established analytical techniques. Molecules in excess of 200kDa have been analysed [16] and electrospray techniques have been widely used for estimating relative molecular masses in a number of proteins (for a review of ES analysis of high-molecular-weight compounds, see Smith 1991) [17].

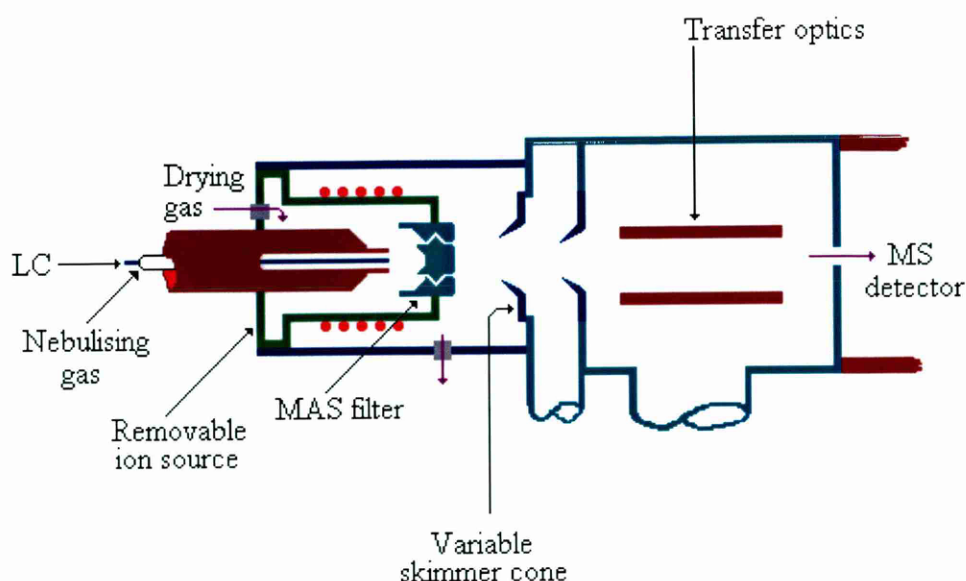


Figure 6.1 Schematic of the electrospray interface for the Platform instrument used for the biological analyses detailed in this Chapter.

The technique of electrospray can be used to determine the relative molecular masses of large analytes, including polymers and small proteins as in the work of Fenn *et al.* [13, 15, 18, 19] who have demonstrated ionization molecules with high relative molecular masses and detection limits in the femtomole range. These analyses are facilitated due to the fact that in the formation of the electrospray many charges are attached to large molecules, thus generating multiply-charged species. In this way a polypeptide that has a mass of 60,000 daltons when carrying 30-60 positive charges will be detected as having a mass-to-charge ratio of 1,000-2,000, and is then within the range of the modern mass spectrometer [20]. The analysis of small molecules such as trace natural and drug metabolites has also been demonstrated [21] including the carnitine esters described here. Figure 6.1 shows a schematic of the electrospray interface used for the analyses described in this chapter.

It was Simons *et al.* who first investigated the coupling of the techniques of electrospray with liquid chromatography (LC-ES/MS) [22]. The interface between the LC and ES

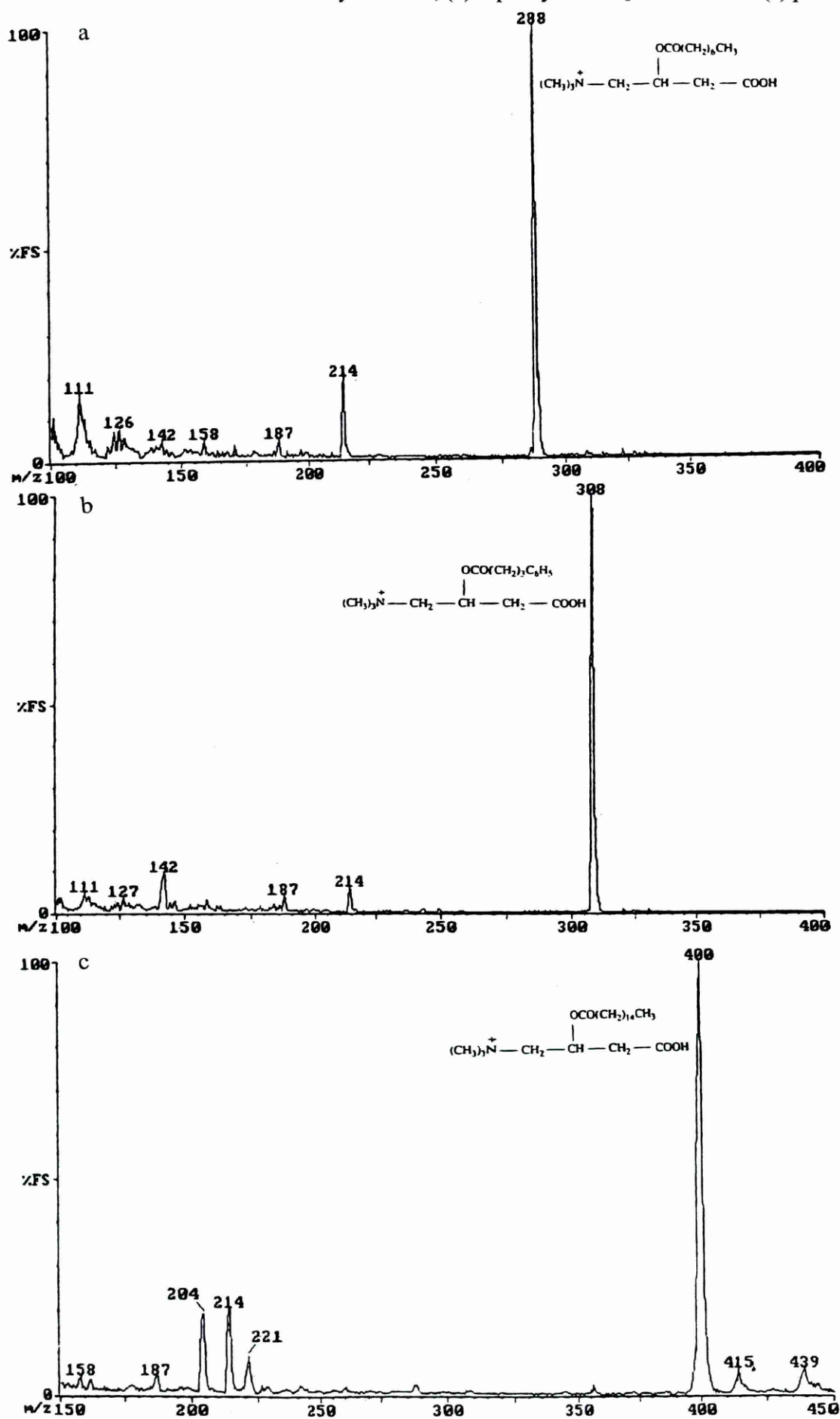
systems is such that a liquid flow, typically at a flow rate of 1-40 $\mu\text{l}/\text{min}$ enters a needle interface which is at high voltage (usually approximately 6 kV). The liquid leaves this needle in the form of the nebulized gas that is desolvated in a nitrogen gas atmosphere, to release the charged ions [20]. The ions are then drawn through a lens and through a cone (skimmer) section and into the region of reduced pressure inside the mass analyser (see Figure 6.1). The nitrogen gas is also responsible for the reduction of ion/solvent clusters formed through hydrogen bonds [23, 24].

6.2 RESULTS AND DISCUSSION.

6.2.1 ANALYSIS OF STANDARD ACYLCARNITINES.

An initial study was carried out to determine if analysis of acylcarnitines was possible using the technique of electrospray mass spectrometry (ES/MS). This initial work has been published [25]. Analysis carried out under the conditions described (Experimental 6.5.1) and presented the preliminary application of positive ion electrospray to underivatized medium and long chain acylcarnitines, as their HCl salts, clearly demonstrating that a number of acylcarnitines of varying chain-length could be analysed. In this case the acylcarnitines investigated included octanoylcarnitine (C_8), the key medium-chain acylcarnitine in the diagnosis of MCAD, and palmitoylcarnitine (C_{16}) a significant metabolite in the diagnosis of LCAD these are shown as Figure 6.2 a and c. In addition to these carnitine esters 4-phenylbutanoylcarnitine was also analysed. This compound is not naturally occurring and this fact and its nature as an acylcarnitine have led to its use as an internal standard in the early work on analysis of acylcarnitines from urine [26, 27].

Figure 6.2 Shows the spectra of standard carnitine esters demonstrating a single strong signal corresponding to the protonated molecule. These are (a) octanoylcarnitine, (b) 4-phenyl butanoylcarnitine and (c) palmitoylcarnitine



Each of the acylcarnitine can be clearly seen to produce a single strong signal, which can be identified by mass spectral information. This is facilitated through the presence of 1 % formic acid in the acetonitrile/water carrier solution allowing the zwitterionic acylcarnitines to exist as cations. In the mass spectra of these compounds the protonated molecule clearly provides the base peak. The electrospray mass spectrum of octanoylcarnitine (Figure 6.2a) consists of the protonated molecule at m/z 288 and a small number of background ions. The mass spectrum of the internal standard, 4-phenylbutanoylcarnitine (Figure 6.2b) consists of a peak from the $[M+H]^+$ ions at m/z 308. The spectrum in Figure 6.2c from analysis of palmitoylcarnitine has the protonated molecule at m/z 400 while the other peaks in this spectrum may be assigned to impurities in the commercial sample including m/z 204, acetylcarnitine. The amount of fragmentation seen using this instrumentation is very small and therefore structural information of any unknown compounds could not initially be obtained. It is however possible to induce fragmentation, thus yielding further information, by increasing in the cone voltage or by collisional activation in a tandem mass spectrometry (MS/MS) experiment.

6.2.2 ANALYSIS OF BIOLOGICAL STANDARD ACYLCARNITINES.

This electrospray study was then extended to the analysis of spiked blood spots from a healthy adult. These samples were prepared as described (Experiments 6.4.2-3) and analysed directly by ES/MS using a 5 μ l loop injection (Experiment 6.4.4). The data obtained from this analysis, clearly demonstrated the presence of octanoylcarnitine. It was the observation of a peak at m/z 288, equivalent to the expected $[M + H]^+$ of octanoylcarnitine which led to this peak assignment. Analysis of samples derived from dried blood spots also repeatedly indicated a second mass peak at m/z 301 at greater intensity than that attributed to octanoylcarnitine (Figure 6.3). The reconstructed ion

Figure 6.3 Electrospray mass spectrum obtained by loop injection of an extracted spiked blood spot sample. Indicating the presence of octanoylcarnitine at m/z 288 and a non-acylcarnitine contaminant at m/z 301.

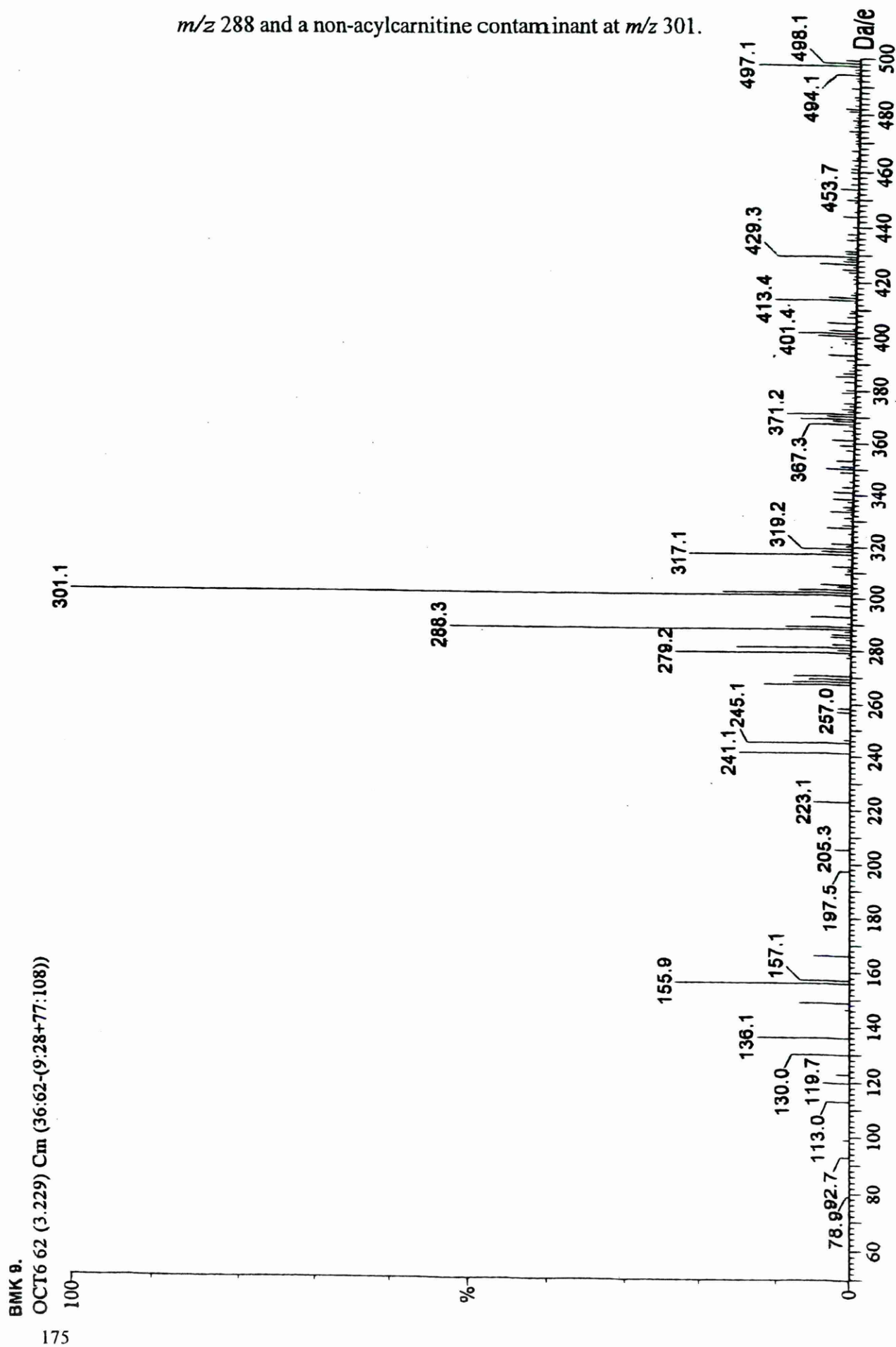
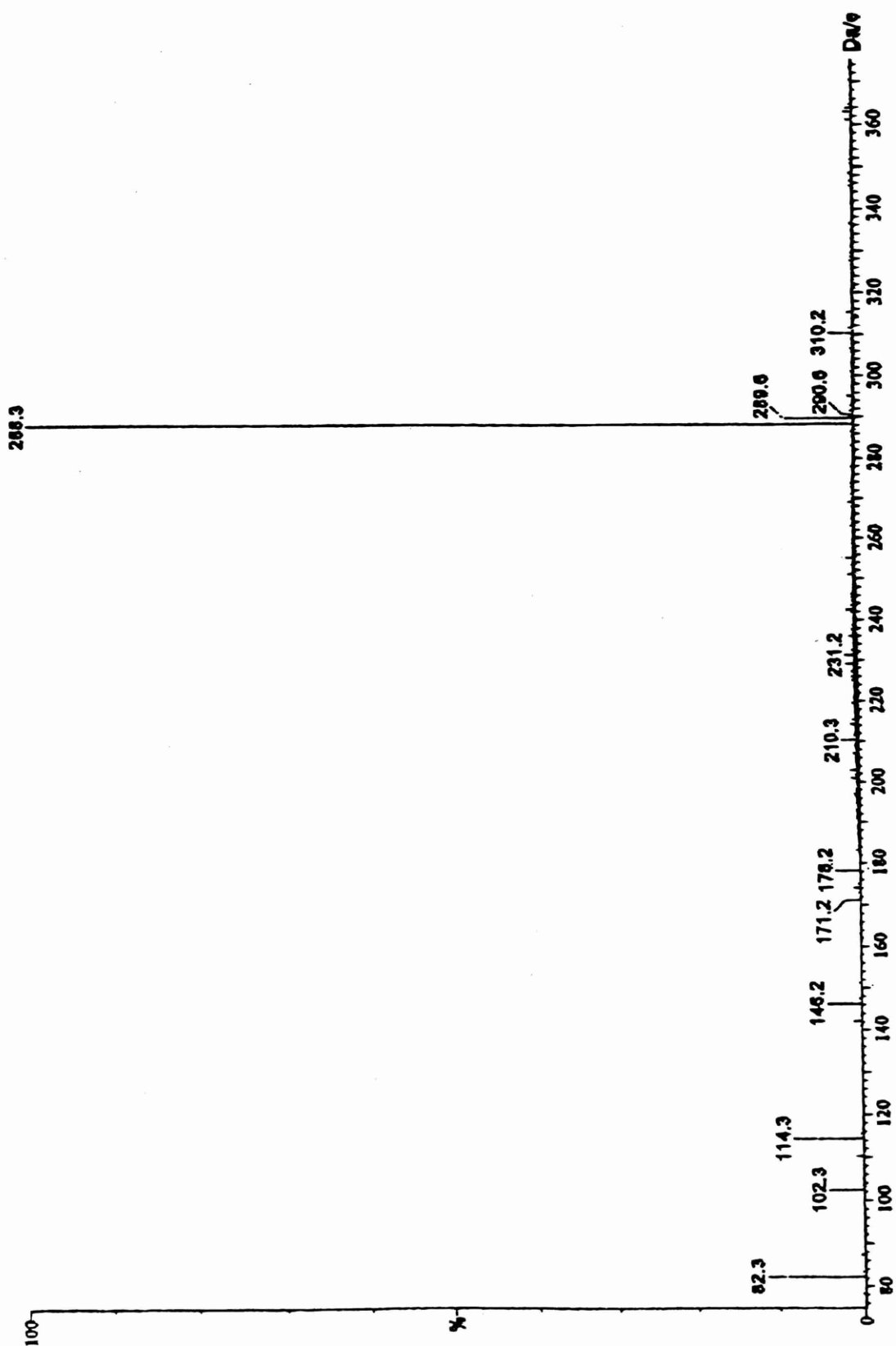


Figure 6.4 Electrospray mass spectrum generated after LC-ES/MS analysis of a spiked blood spot sample. The base peak is now the $[M + H]^+$ of octanoylcarnitine, m/z 288

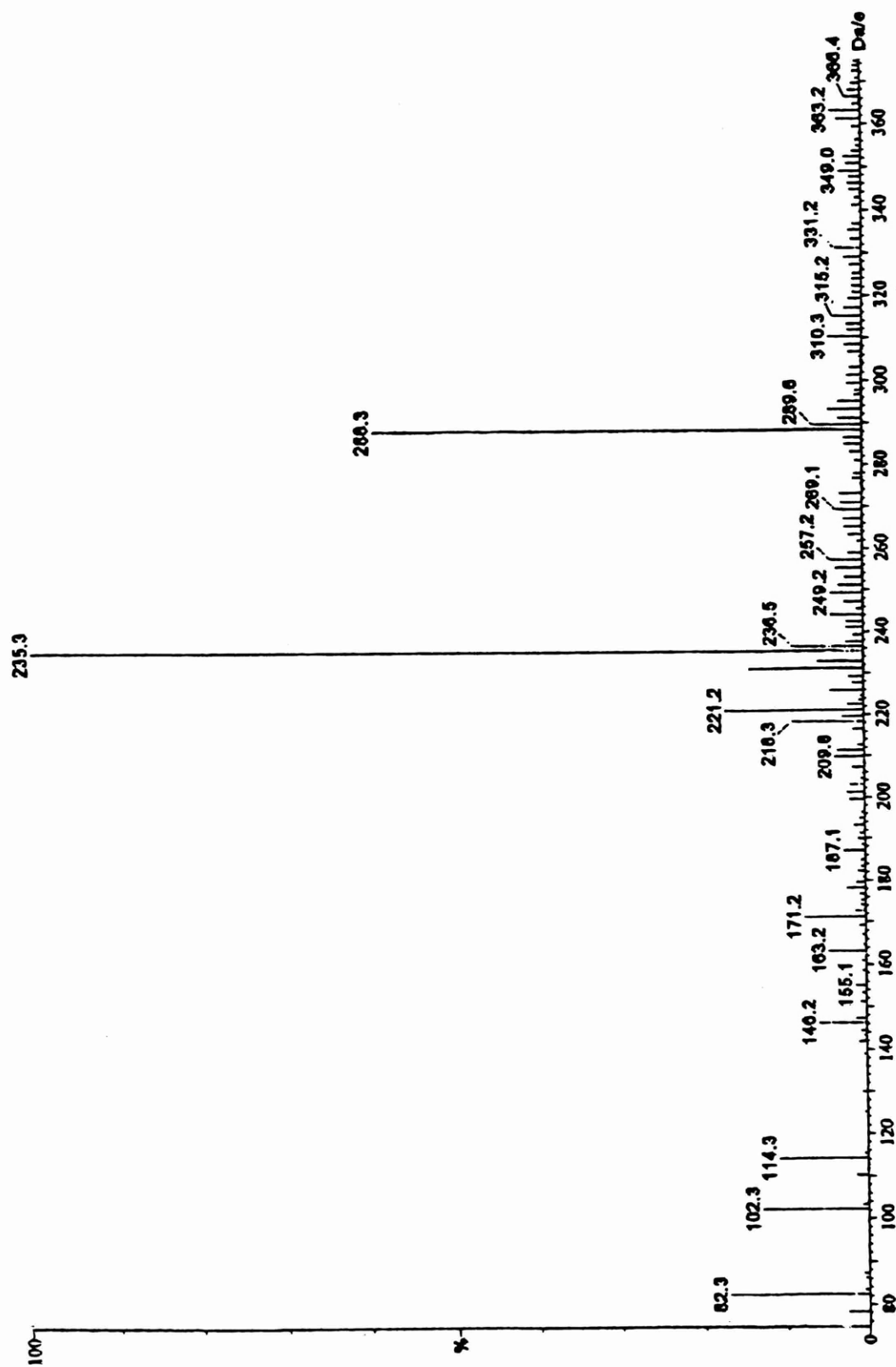


current of this sample was generated and the profiles clearly indicated that the source of both m/z 288 and 301 was the same.

The fact that the contaminating peak had an odd m/z value indicated that it was not an acylcarnitine but could be derived from the blood matrix as its profile indicated its source as the injected sample. It was considered that the addition of a separating technique prior to ES/MS might remove this unidentified contaminating compound. Liquid chromatography was the technique chosen as this is easily coupled with the electrospray instrumentation as described above and the flow rates allowing carrier solvents and analytes to enter the source directly without any splitting or manipulation being necessary. The carrier solvent of acetonitrile/water meant an identical sample could be used as for the initial loop injected samples.

The chromatographic properties of standard non-biological octanoylcarnitine were first investigated followed by analysis of spiked blood spot using LC-ES/MS. The liquid chromatography separation was accomplished using an Applied Biosystems, Aquapore RP-30 column and the eluted carrier solvent and analyte was then fed to the electrospray source directly, as outlined in experiment 6.4.4. The retention times for both the standard and the extracted octanoylcarnitine were identical and reproducible, at approximately 18.5 min. The use of the separation procedure was also successful in eliminating the contaminant peak from the mass spectrum of octanoylcarnitine from spiked blood spots (Figure 6.4), though the compound giving rise to the m/z 301 was not identified by LC-ES/MS. There was however a peak that eluted approximately 2.5 min after the octanoylcarnitine in all extracted samples and has been attributed to a non-acylcarnitine component of blood. The mass spectrum of this unknown peak suggests a relative molecular mass of 278 as its $[M + H]^+$ ion is recorded at m/z 279.

Figure 6.5 Spectrum resulting from the ESI/MS analysis of an MCAD sample



6.3 APPLICATION TO CLINICAL SAMPLES.

Blood spots on Guthrie cards were obtained from a number of patients all of whom had been diagnosed as suffering from inherited metabolic disorders. Most of these samples are duplicates of samples previously described in Chapter 5 and therefore only a brief introduction to each will be outlined here. Analysis was carried out in all cases by both ES/MS loop injections and with addition of a separation stage in the form of LC-ES/MS.

6.3.1 MEDIUM-CHAIN ACYL-CoA DEHYDROGENASE DEFICIENCY (MCADD).

Medium-chain acyl-CoA dehydrogenase (MCAD) is one of a three mitochondrial matrix acyl-CoA dehydrogenases which carry out the initial dehydrogenation step in the β -oxidation of straight chain fatty acids. Its role in disease is discussed in Section 5.7 Chapter 5 of this thesis.

The diagnostic compound for MCADD was the medium-chain acylcarnitine, octanoylcarnitine, as investigated above. Initially a direct ES/MS analysis was carried out on this sample by loop injection the results of which are shown in Figure 6.5. This indicated the presence of octanoylcarnitine within the residue mixture, due to the observed ion at m/z 288 at a significant relative intensity, but as with spiked samples described above incorporation of a separation step was necessary in order to generate a clearer electrospray mass spectrum. Applying the procedure of liquid chromatography the retention time of standard octanoylcarnitine was found to be reproducible, approximately 18.5 min. Analysis was performed using an Aquapore RP-30 and the conditions (Experiment 6.4.4) included gradient elution at 40 μ l/min using a linear gradient from 95% 0.01M ammonium acetate, 5% methanol to 100% methanol over 20 min with the eluant introduced directly to the electrospray source. After injection of an analytical blank, an

identical analysis was carried out on an MCADD sample. Octanoylcarnitine was determined to be present through chromatographic and mass spectral information. The retention time was again approximately 18.5 min and a significant response at m/z 288 was evident, thus confirming the diagnosis of medium-chain acyl-CoA dehydrogenase deficiency on the basis of increased excretion of octanoylcarnitine. From the mass spectrum and a reconstructed ion current, it is proposed that the additional component, producing the base peak at m/z 235, co-elutes with octanoylcarnitine as it has an identical profile in the chromatogram produced. At present the compound responsible for this co-eluting peak remains unidentified.

It has therefore been clearly shown that octanoyl carnitine, a diagnostic marker for MCADD was present in this sample. Although a loop injection was sufficient to identify octanoylcarnitine the chromatographic step prior to detection allows greater confidence in its identification. The analysis of a standard followed by a blank and then the clinical sample excludes any within day variability in the assay and also the potential for the production of a false positive result due to carry-over.

Therefore we have shown that ESI-MS can be used to characterise an MCADD sample from the presence of octanoylcarnitine in neonatal blood spots.

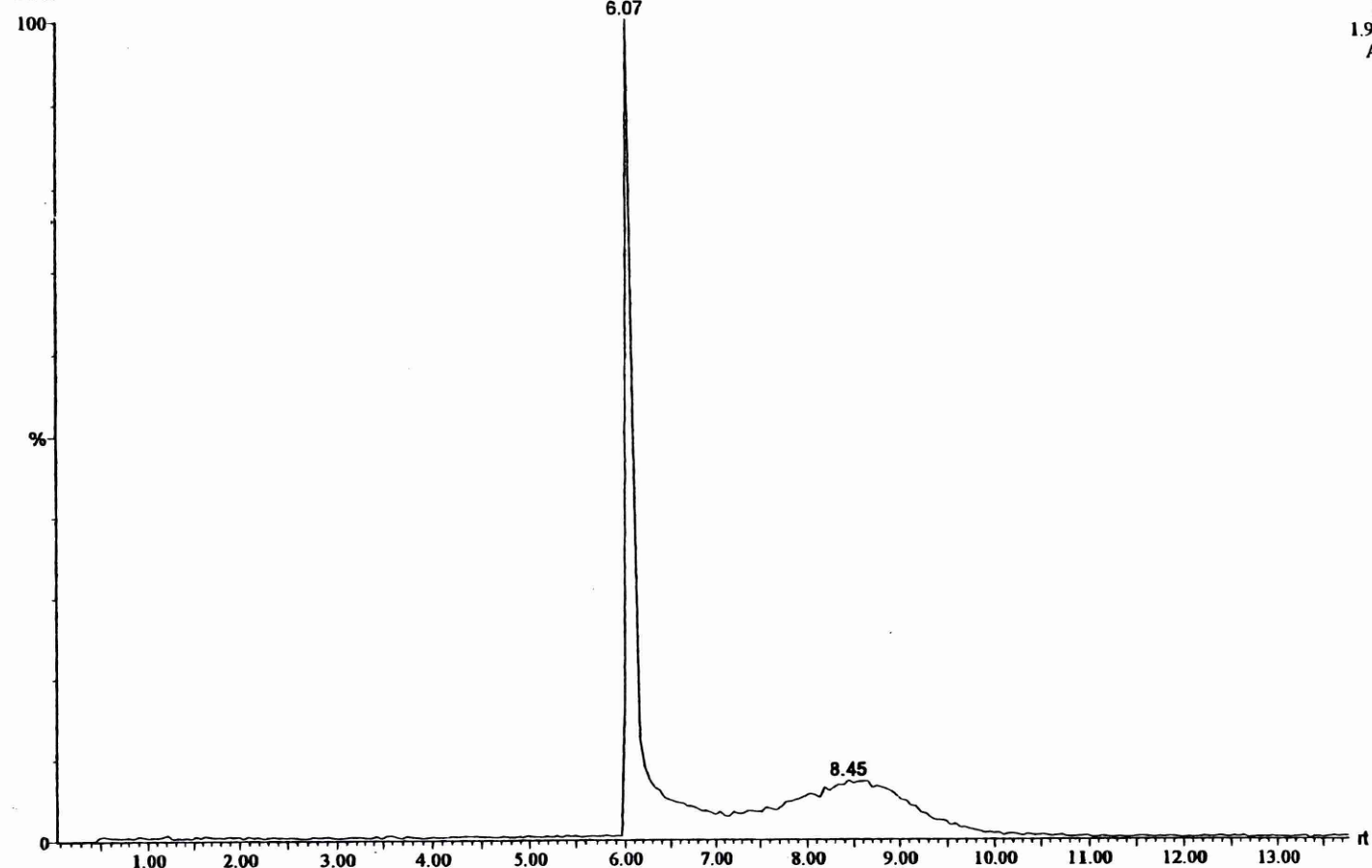
6.3.2 PROPIONIC ACIDEMIA

In the disease propionic acidemia (PA) the major urinary metabolite is reported as 2-methylcitrate [28, 29, 30] with the disease characterized by severe metabolic decompensation with metabolic acidosis and hyperammonaemia. The role of propionic acid in this disease is discussed in Section 5.7 Chapter 5 of this thesis.

Figure 6.6 Chromatogram of standard solution of propanoylcarnitine (above) and spectrum showing m/z 218 (below)

Propinoyl carnitine.

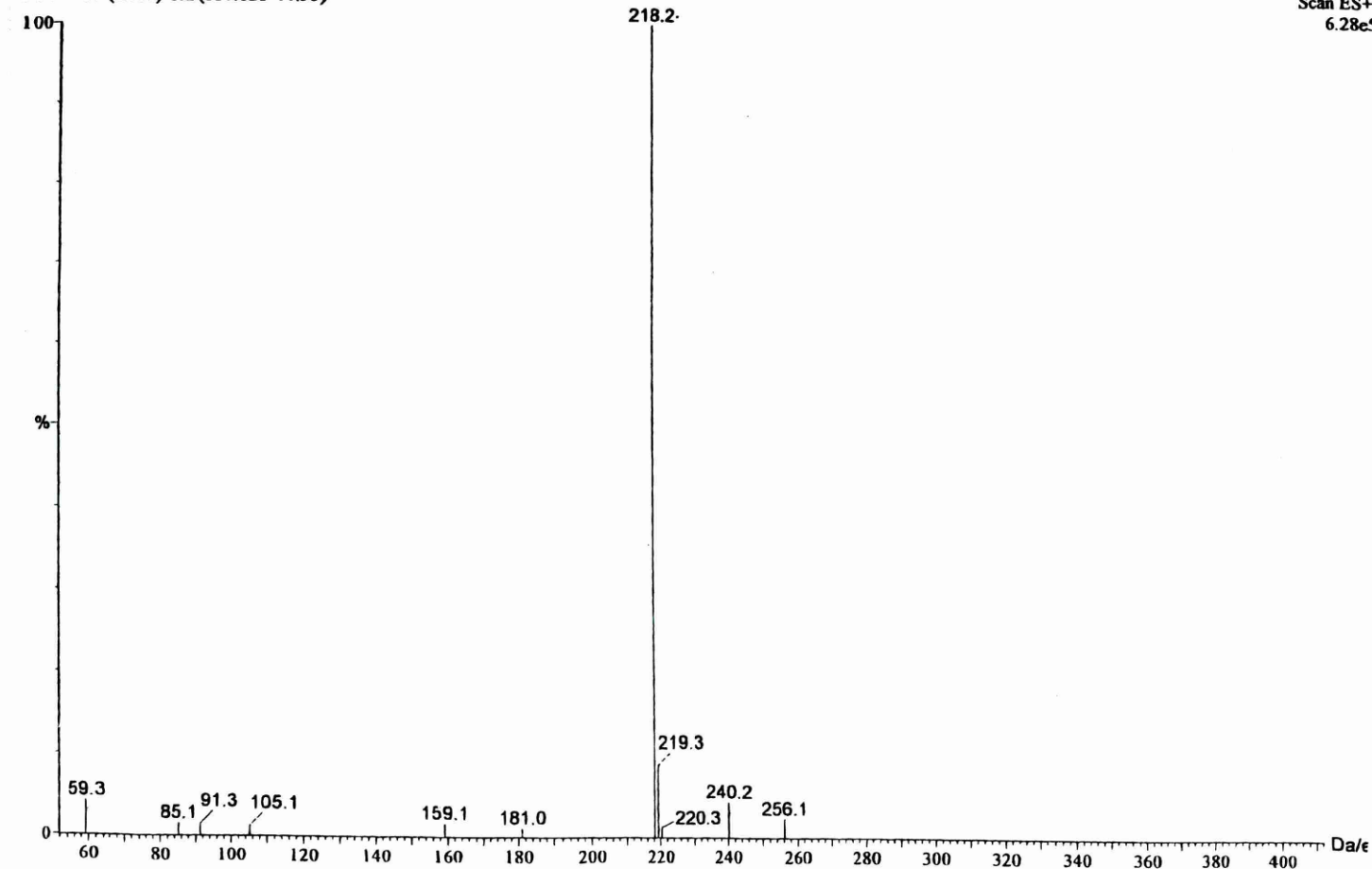
OU15



-Scan ES+
218
1.91e6
Area

Propinoyl carnitine.

OU15 117 (6.070) Cm (116:121-44:95)



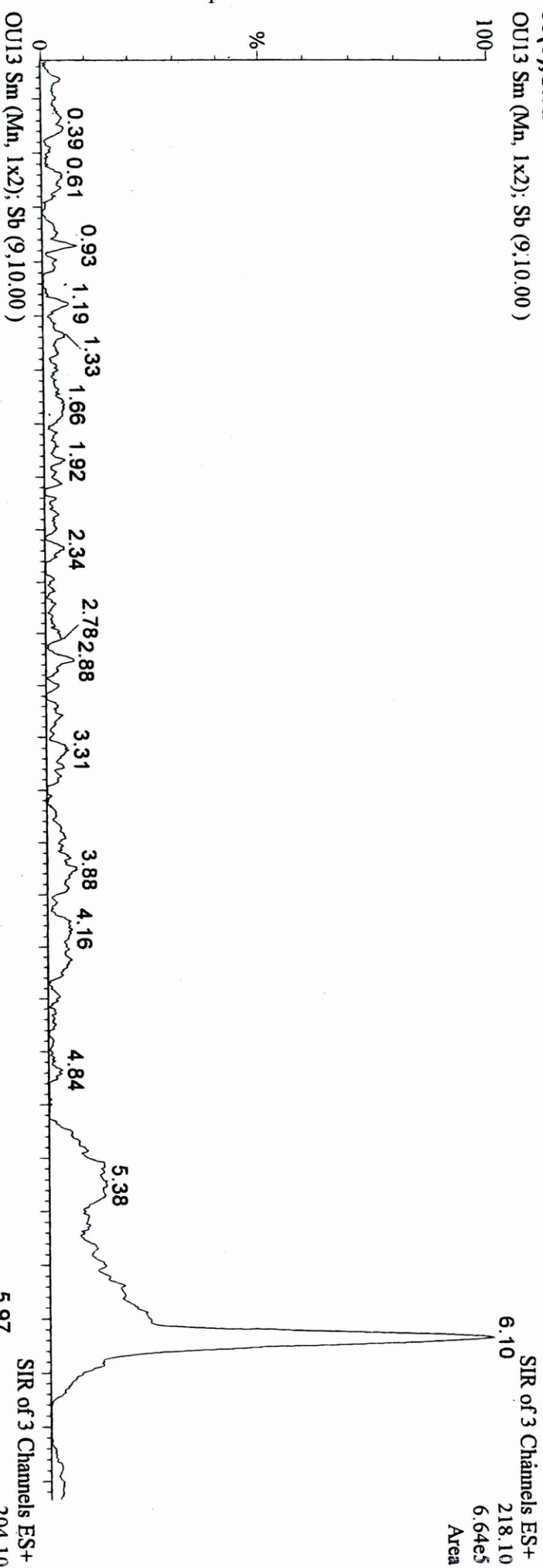
Scan ES+
6.28e5

In the ES-MS and LC/ES-MS study of a patient diagnosed as suffering from the disorder propionic acidemia the aim was the detection of propanoylcarnitine (m/z 218), the sample was prepared as described in Experiment 6.4.2-3. The result from analysis of a standard is shown in Figure 6.6. The initial analysis was carried out via a loop injection of 5 μ l directly by ES-MS. The results obtained in this way clearly demonstrated the presence of the required protonated molecule, at approximately 50% relative abundance, within the residue mixture. Within this matrix it is also proposed that the protonated molecules of free carnitine (m/z 162) and acetylcarnitine (m/z 204) were present as would be expected from this medium.

LC-ESI/MS was carried out as described (Experiment 6.4.4) using a Phase Separation 1 mm x 25 cm C8 column. The change of column was due to the fact that the Aquapore RP-30 used in the MCADD study (Experiment 6.4.4) is considered more suitable for the analysis of proteins and large molecular weight molecules whereas the column used here was considered more suitable for the smaller acylcarnitines and would therefore potentially improve peak resolution. An initial study was carried out to determine the retention time of propanoylcarnitine and also that of acetylcarnitine which also demonstrated that they eluted close together with retention times of 6.10 and 5.97 minutes respectively, as shown in Figure 6.7. The clinical sample diagnosed as propionic acidemia (PPA) was then analysed via this method.

65(6),SIR.

OUI3 Sm (Mn, 1x2); Sb (9,10.00)



SIR of 3 Channels ES+
204.10
3.51e5
Area

SIR of 3 Channels ES+
218.10
6.64e5
Area

Figure 6.7 Propionic acidemia clinical sample

The resulting total ion chromatogram (TIC) indicated the presence of both acetyl and propanoylcarnitine as seen from the loop injection. Though it was necessary to reconstruct the profiles of the protonated molecules of interest to determine if either was significant from the noise level. Both were significant though with poor peak shape in the expected retention time window though again with similar retention times (acetylcarnitine 5.97 min, propanoylcarnitine 6.10 min). A single ion recording (SIR) was also carried out which served to confirm the identity of the compounds and the reproducibility of the separation.

6.3.3 METHYLMALONIC ACIDEMIA.

Methylmalonic acidemia has been shown to be caused by the absence or deficiency of methylmalonyl-CoA mutase or by abnormalities of intramitochondrial cobalamin metabolism [31]. Its incidence has been reported as 1 in 48,000 births in one screening program [32].

Preparation and analysis of the dried blood spot from a patient with diagnosed methylmalonic acidemia was carried out in an identical fashion to that of the PA sample described above (Section 6.3.2). In the case of this disorder one of the important diagnostic peaks is from the detection of propanoylcarnitine as in the case of PA. There is however a second metabolite which may be detected in this disorder, methylmalonylcarnitine (m/z 262), a medium branched-chain acylcarnitine derived from the acid of the same name. It is detection of this second metabolite that would allow unambiguous determination of this disorder, avoiding confusion with PA.

With the chromatographic and mass spectral nature of propanoylcarnitine established from above (Section 6.3.2), detection in the case of the methylmalonic acidemia sample was

carried out in an identical manner with the positive identification of the metabolite propanoylcarnitine. Though the sample was also analysed for the presence of methylmalonylcarnitine the expected response at m/z 262 was not detected.

In summary, a diagnosis of a disorder of fatty acid metabolism could be made from the above analysis and a proposal of propionyl or methylmalonic acidemia made.

Unfortunately, the inability to detect the methylmalonylcarnitine that would confirm the latter disorder would mean that further characterisation of this sample would be necessary.

In conclusion, the experiments outlined in this chapter are a small insight into what will no doubt become an important area in the diagnosis of metabolic disorders. We have shown the potential of this technique for the identification of acylcarnitines with very little sample work-up prior to analysis. Though the LC separation is at present a time consuming step prior to detection it provides a vital sample clean-up and while it should not be excluded there is the potential for shortening the run times while maintaining separation where more than one acylcarnitine is being detected. The use of MS/MS may aid the shortening of this run time by affording greater selectivity and thus reducing the necessity for chromatographic separation of analytes and this should be investigated further particularly in the light of more recent work (see Chapter 7).

6.4 EXPERIMENTAL.

6.4.1 PREPARATION OF STANDARDS.

The acylcarnitines, as hydrochloride salts, were either obtained from the Sigma Chemical Company, UK (dl-octanoylcarnitine. HCl) or prepared by an established method [36]. Electrospray mass spectrometry of non-biological standards was carried out on a VG Trio-2000 operating with a cone voltage of about 30 V. The carrier solution was acetonitrile/water (50:50) containing 1% formic acid and its flow rate was 5 µl/min.

6.4.2 PREPARATION OF BIOLOGICAL STANDARDS.

Fresh blood (10 ml) was stored in a flask of solid carbon dioxide for the shortest time possible (5-10 min). Spiking of the blood was carried out by the addition of varying concentrations of octanoylcarnitine solution (as Chapter 5, Table 5.1) to 0.5 ml aliquots of fresh blood. The samples were then shaken and spotted onto Guthrie paper and stored at room temperature.

6.4.3 EXTRACTION OF ACYLCARNITINES FROM DRIED BLOOD SPOTS.

Acylcarnitines were recovered from dried blood spots by sonicating with a solvent. A circular spot of 6 mm diameter was punched out and extracted twice with a chloroform/methanol mixture (2:1, 2 x 1 ml), sonicating each time for 10 min. Spiked spots contained down to 200 pmol of the octanoylcarnitine. The combined extracts were then dried and the residue dissolved in 50 µl acetonitrile/water for electrospray analysis.

6.4.4 INSTRUMENTATION AND ANALYSIS CONDITIONS FOR BIOLOGICAL SAMPLES.

Electrospray mass spectrometric analyses of samples of biological origin were performed on a VG Platform, benchtop, single quadrupole mass spectrometer (Fisons Instruments/VG BioTech).

Loop injections were performed using a Rheodyne 8125 injector with a 5 μ l loop.

Injection volume was 5 μ l from the sample residue in 50 μ l acetonitrile/water. LC/MS was carried out using an Applied Biosystems, Aquapore RP-30, 100 x 1.0 mm column.

Gradient elution at 40 μ l/min was performed using a linear gradient from 95% 0.01M ammonium acetate, 5% methanol to 100% methanol over 20 min with the eluant introduced directly to the electrospray source. Some LC/MS analyses were performed using a Phase Separation 1 mm x 25 cm C8 column with acylcarnitines isocratically eluted at 100% 0.01M ammonium acetate for 5 min followed by a ramp to 100% methanol, with introduction into the source as above.

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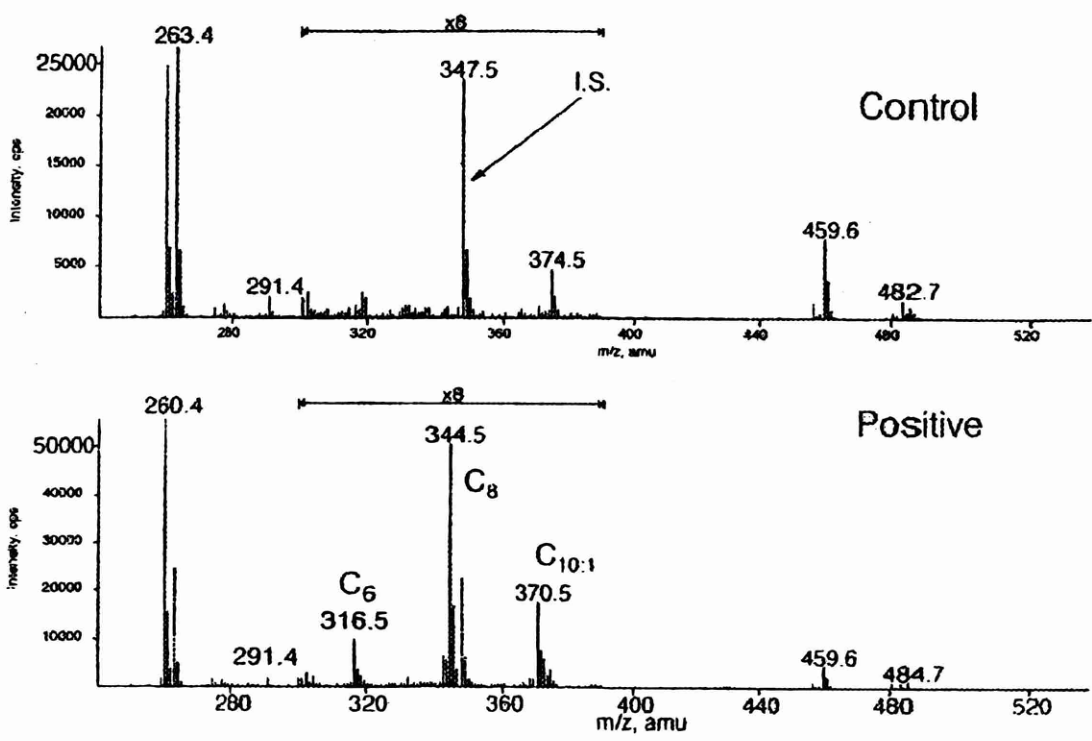
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CHAPTER 7

CONCLUSION AND FUTURE WORK

Figure 7.1 MS/MS acylcarnitine profile obtained with a precursor of m/z 85 scan function from a healthy newborn (top) and the screening card of a newborn with MCADD [3]



7.1 ADVANCES IN ESI-MS – EXPLOSION IN TANDEM

In the time since the conclusion of the practical work outlined in this thesis it is the area of electrospray mass spectrometry, particularly with the use of tandem mass spectrometry, has continued to develop at the most rapid rate.

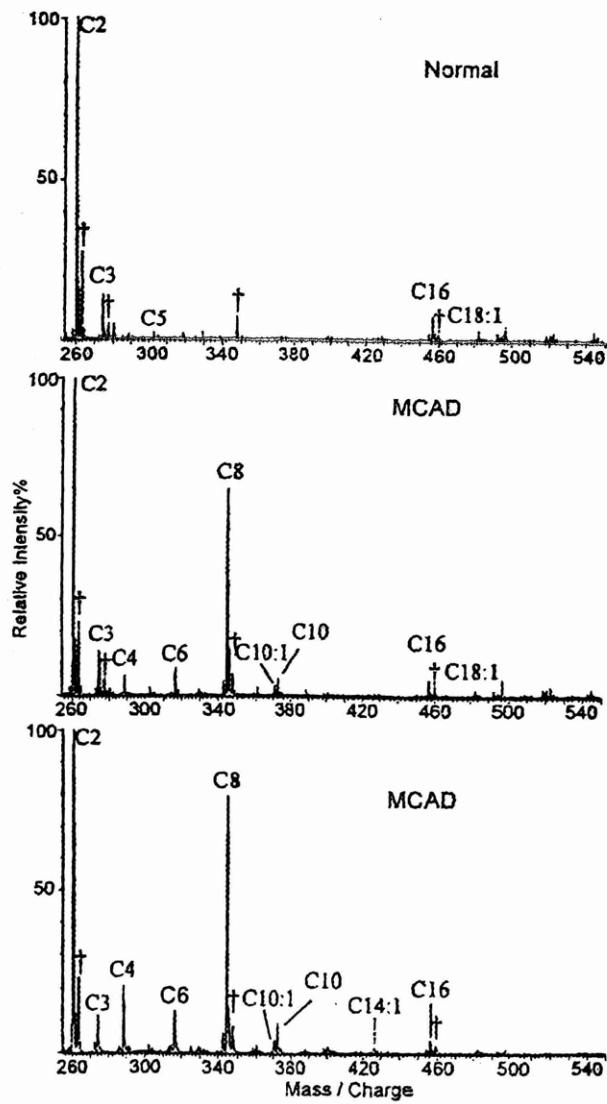
The purpose of any screening technique is to provide an early diagnosis, which will enable early medical intervention and prevent or reduce clinical symptoms such as mental retardation. The criteria to allow for screening to be introduced are as follows [1]. The disorder has to have a relatively high incidence so that the cost per diagnosis is reasonable. The test must be relatively inexpensive and suitable for high volume testing. The test must have both high sensitivity and selectivity.

The area of screening and metabolic profiling for both amino acids and acylcarnitines, using differing scan functions on the mass spectrometer, has meant that the satisfaction of just some of the criteria above is no longer the aim of a technique. To be both commercially and medically acceptable any technique must aim to address all.

Since early workers in this field with success in the area of FAB-MS/MS reported the diagnosis of PKU from neonatal blood spots [2] advances have continued at a rapid rate. This technique, also reported as static liquid secondary ionisation (LSI) – MS/MS, is now routinely used particularly in Pennsylvania and North Carolina where screening has shown a disease frequency of 1 in 17,706 for MCAD [3]. Figure 7.1 shows MS/MS acylcarnitine profiles obtained using this technique for normal versus MCAD patients.

The use of ESI-MS/MS is also now widely reported in the literature in many cases also making use of advances in instrument automation such as auto-injection [4] and

Figure 7.2 Ionspray-MS/MS spectrum from an MCAD affected neonate (two lower spectra) and that of a control subject (top) using a precursor ion scan of m/z 85 [5]



microplates with computer flagging of abnormal profiles of both amino acids and acylcarnitines [5].

The MS/MS techniques available now have the facility to carry out more complex scanning than previously. For example, using ionspray (a form of electrospray) coupled with MS/MS, a single test with an analysis time of approximately two minutes can be used to determine levels of amino acids and acylcarnitines from a single blood [6]. By screening for precursors of m/z 85 and a neutral loss of 102, alternating scan types, disorders of fatty acid metabolism can be determined. These are derived from the butyl esters of acylcarnitines and free amino acids [2, 5]. Using this technique quantitation of amino acid and acylcarnitine levels has been possible for known indicators of approximately 25 inherited metabolic disorders in. Examples of some of the data produced using this technique are illustrated in Figure 7.2 and Table 7.1 with the latter presenting the breadth of diagnoses possible in neonates [6].

Table 7.1 - Illustrates the breadth of diagnoses possible in neonates using MS/MS [6]

Metabolic Disorder of ...	Disease
Amino Acids	PKU
	MSUD
Organic Acids	Propionic acidemia
	Isovaleric acidemia
Fatty Acids	LCHAD
	3-methyl crotonyl CoA Carboxylase deficiency

Over a few short years we have therefore seen the development of this technique and other tandem MS techniques become more viable for use as screening procedures. By the criteria outlined earlier this technique can provide a sensitive and selective analysis tool. The potential for the diagnosis of multiple disorders and the reported sample analysis time makes the cost per sample viable despite the high cost of the instrumentation required.

Overall electrospray coupled with mass spectrometry has become a bright light in the arena of metabolic disorders.

7.2 CONCLUSION

The work that has been presented in this thesis has built on the overall aim of providing a technique, which is within the reach of the widest range of the paediatric medical profession possible. The hope is that with existing instrumentation a hospital might provide a facility, which could save lives. Perhaps, not through population screening, as is the aim of some of the more expansive and expensive techniques, but through the screening of siblings and other babies at risk. Preventing further distress to families or in providing an answer to the question of why a child had unexpectedly died.

What we have succeeded in demonstrating are a number of applications of a basic method. We have shown (in Chapter 3) how advances in more routine laboratories have enhanced the ability to characterise acylcarnitines in urine and through being able to confirm the presence of a wide variety of acylcarnitines a diagnosis may be aided. The value of this extra level of confidence cannot be underestimated in an area where the correct result is the only possible one and false positives or indeed negatives could cause so much damage.

In Chapter 4 we attempted to extend the method to the analysis of dicarboxylic acylcarnitines. Synthesis and analysis of these compounds has been possible though further work is required to understand at which point the lactonisation is failing further work and development in this area is required to broaden the scope for diagnosis.

Other work, which broadens the application for this method is outlined in Chapter 5 with the analysis of blood spots from Guthrie cards. The fact that blood spots are easily available and

long-term storage is not an issue, are important reasons to view this area as a huge advancement over urine analysis. There is also the feature that blood spots have traditionally been acquired, and are accepted, as a sample required from newborns. Approaching a mother for a further urine sample may beg the question as to why it is required and raise unnecessary anxiety. A method has been presented here and its development detailed. Also, a number of clinical samples from a variety of fatty acid disorders have been analysed in order to test the method with 'real' samples.

While the limited amount of quantitative work suggests that the recovery from the extraction and lactonisation is low the clinical sample results suggest sufficient sensitivity is be present to allow diagnosis, due to the high levels of the marker acylcarnitines and their distinctive characterisation by mass spectrometry as lactones.

Finally, a foray was made into the world of electrospray. This proved very fruitful in demonstrating the powerful tool, which was at the time becoming more widely available to analysts. A review of the literature today will quickly show applications for this technique in many fields of science. In the area of neonatal metabolic disorders it has been shown that the initial very high cost, if possible to obtain, can be offset by sample speed and throughput in the true neonatal screening situation as outlined in the final section of Chapter 6. Our work demonstrated the ease of analysis without derivatization either to the lactone, which is unnecessary as volatility is not an issue, or to butyl esters which features in many other literature methods discussed in Chapter 6.

7.3 FUTURE WORK

There are a number of issues from the work outlined in this thesis which would merit a revisit if further work were to be carried out in this area.

The method in place for the analysis of acylcarnitines in urine is a very robust one and in itself should remain as is. In this case technology, which continues to advance apace will no doubt increase the breadth of what we see and may further enhance the diagnoses possible. The analysis of dicarboxylic acylcarnitines would be an impressive weapon in our diagnostic armoury if it were achievable and this is possibly the area which would provide the greatest challenge to those willing to accept it.

As with the analysis of urine samples, technology will provide an improvement to our ability to confirm disorders of fatty acid metabolism by GC-MS in blood spots as technology continues to advance. To return to the area of recovery of the acylcarnitine lactones from blood spots and to understand which point of this procedure the recovery is retarded could be another aim of future work. Fine-tuning of the method might improve the recovery, which would add an extra degree of confidence to the diagnosis perhaps through the identification of a greater number of diagnostic compounds in samples.

With a view to the wider field of diagnosis of metabolic disorders in neonates areas such as immunoaffinity based screening show great potential. In this case a spot may be extracted with water and the supernatant added to a 96-well plate. The antibody-analyte is then eluted with methanol, derivatized and analysed by MS/MS. Pre-natal cell diagnosis is also under investigation, where fatty acids labelled with stable isotopes are administered. Fibroblasts grown from these can then be analysed i.e. for the presence of labelled acylcarnitines [7]. Other methods include the use of nucleic acid probes [8] or the measurement of the rate of CPT1 and from this the levels of acylcarnitines [9]. A molecular diagnosis has also been investigated through amplification of a genomic DNA segment containing known mutations and cleavage of either normal or variant strands by endonucleases. In this way microgram quantities of DNA can be made available for analysis and even point mutations detected [10].

Finally, with such great advances in mass spectrometry and other techniques it would be almost impossible not look around, as a child in a sweet shop, at the vast array of shiny new things available in the laboratory and to have a small taste of each

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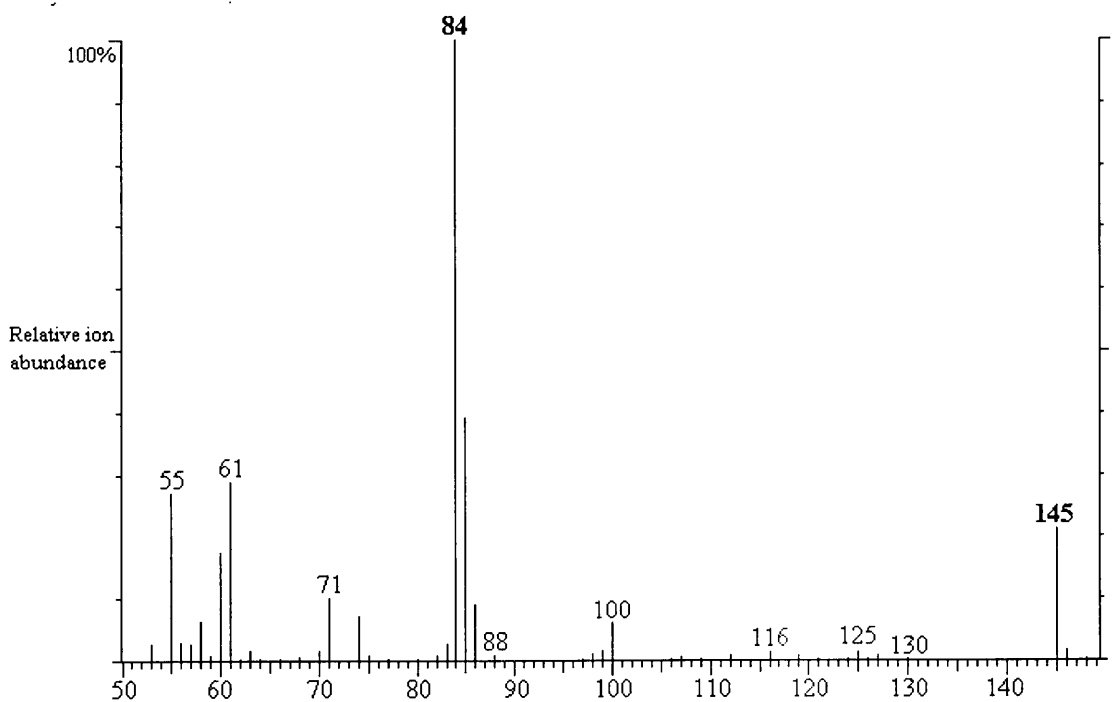
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- 2 D.H. Chace, D.S. Millington, N. Terada, S.G. Kahler, C.R. Roe and L.F. Hofman, *Clin. Chem.* **39(1)**, 66-71 (1993).
- 3 D.H. Chace, S.L. Hillman, J.L.K. Van Hove and E.N. Naylor, *Clin. Chem.* **43** (11) 2106 – 2113 (1997).
- 4 M.S. Rashed, P.T. Ozand, M.P. Bucknall and D. Little *Pediatr. Res* **38(3)**, 324 – 331 (1995)
- 5 M.S. Rashed, M.P. Bucknall, D. Little, A. Awad, M. Jacob. M. Alamoudi, M. Alwattar and P.T. Ozand, *Clin. Chem* **43(7)** 1129 1141 (1997).
- 6 M. Chan, MDS Sciex, Application Note 1998.
- 7 D.S. Millington, presentation at the 23rd BMSS Meeting, University of Warwick 1998.
- 8 P. Ross, K. Lee and P. Belgrader, *Anal. Chem.* **69**, 4197-4202 (1997)
- 9 Nolte *et. al* *Anal Biochem* **256**, 178 (1998).
- 10 Y. Wada, *J. Mass Spectrom.* **33**, 187 (1998).

APPENDIX A

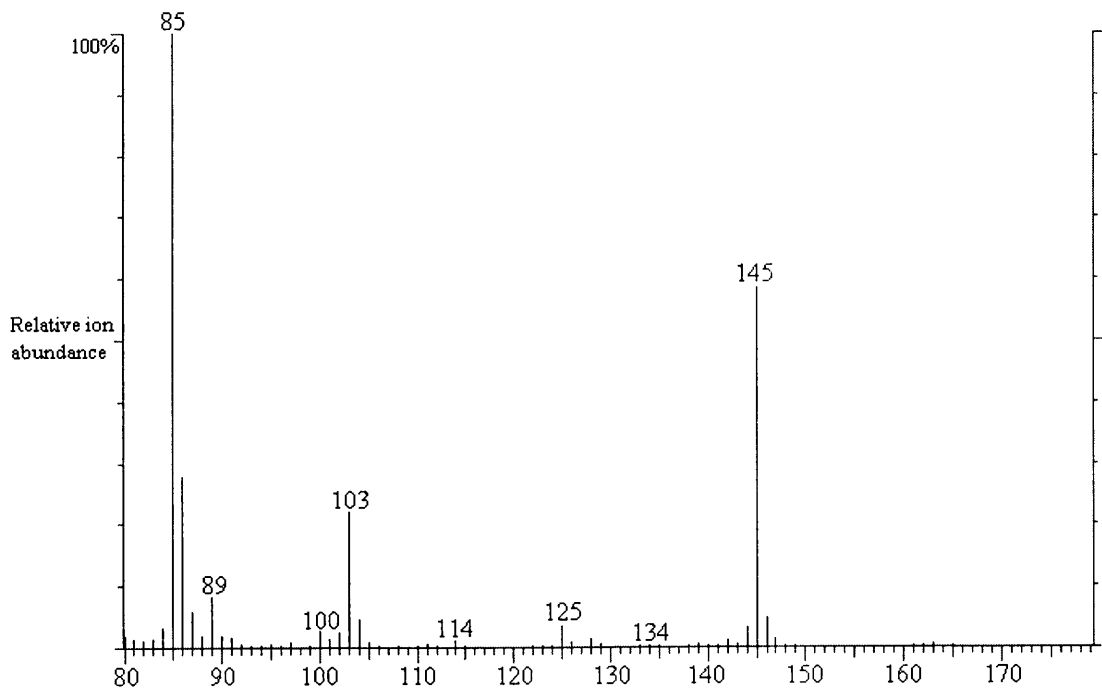
SUMMARY OF APPENDIX CONTENTS

A1	Acetylcarnitine lactone
A2	Propanoylcarnitine lactone
A3	Isobutanoylcarnitine lactone
A4	2-Methylbutanoylcarnitine lactone
A5	Isovalerylcarnitine lactone
A6	Hexanoylcarnitine lactone
A7	An octenoylcarnitine lactone
A8	Octanoylcarnitine lactone
A9	4-Phenylbutanoylcarnitine lactone (Internal standard)

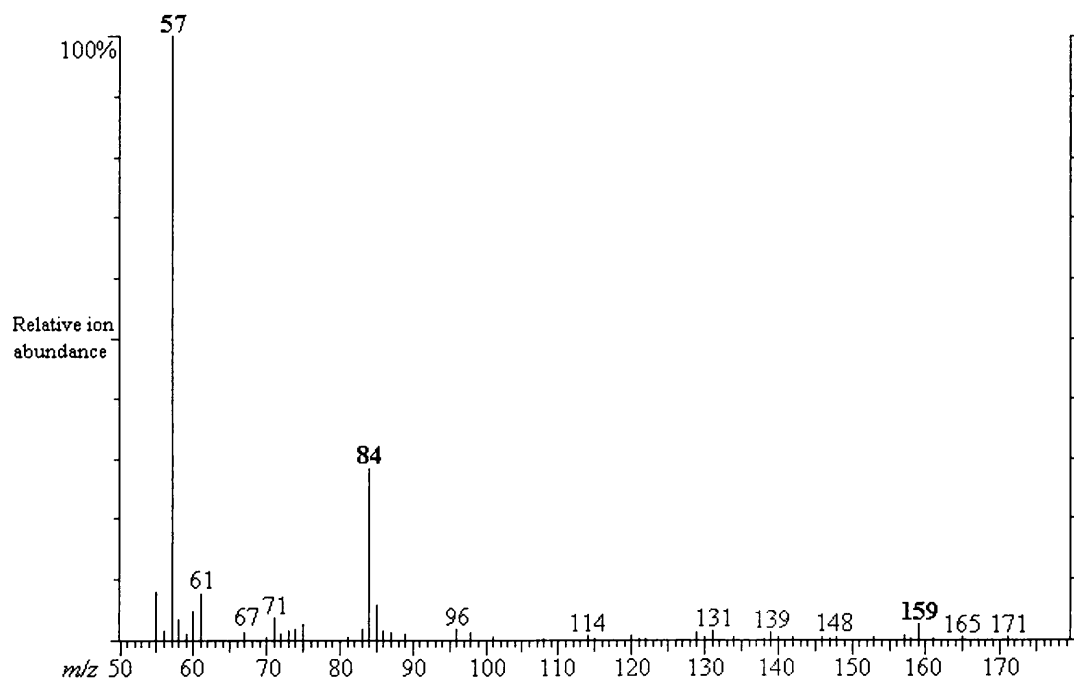
A1 MADD urine sample,
Acetylcarnitine lactone (scan number 331, Figure 3.3), EI mode.



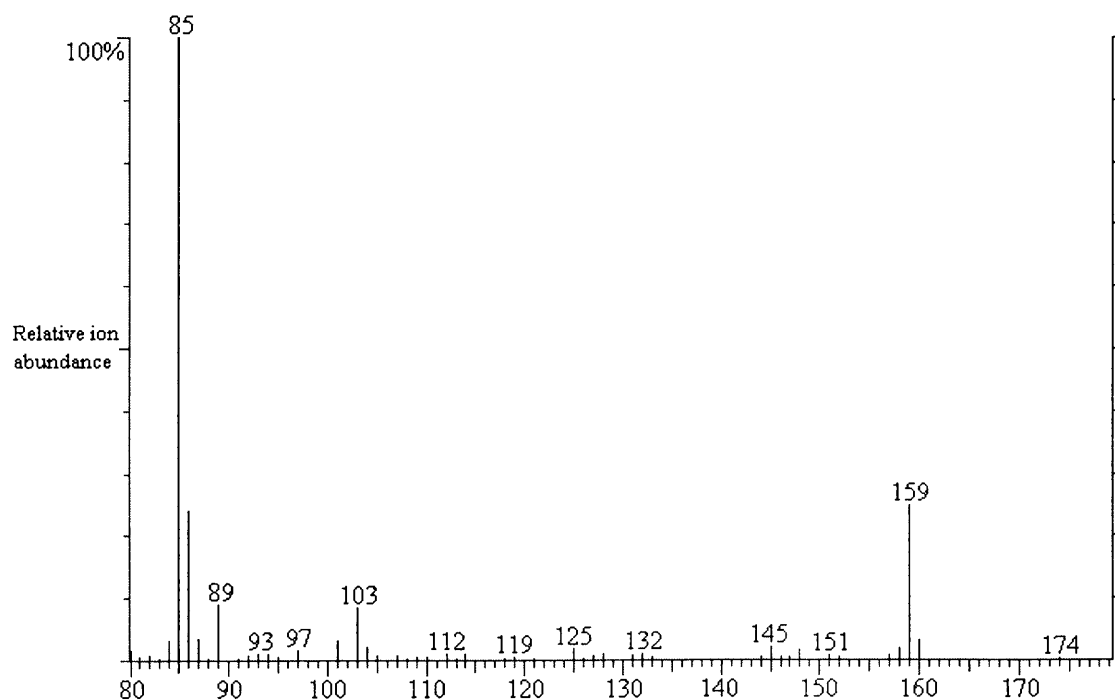
Acetylcarnitine lactone, CI mode.



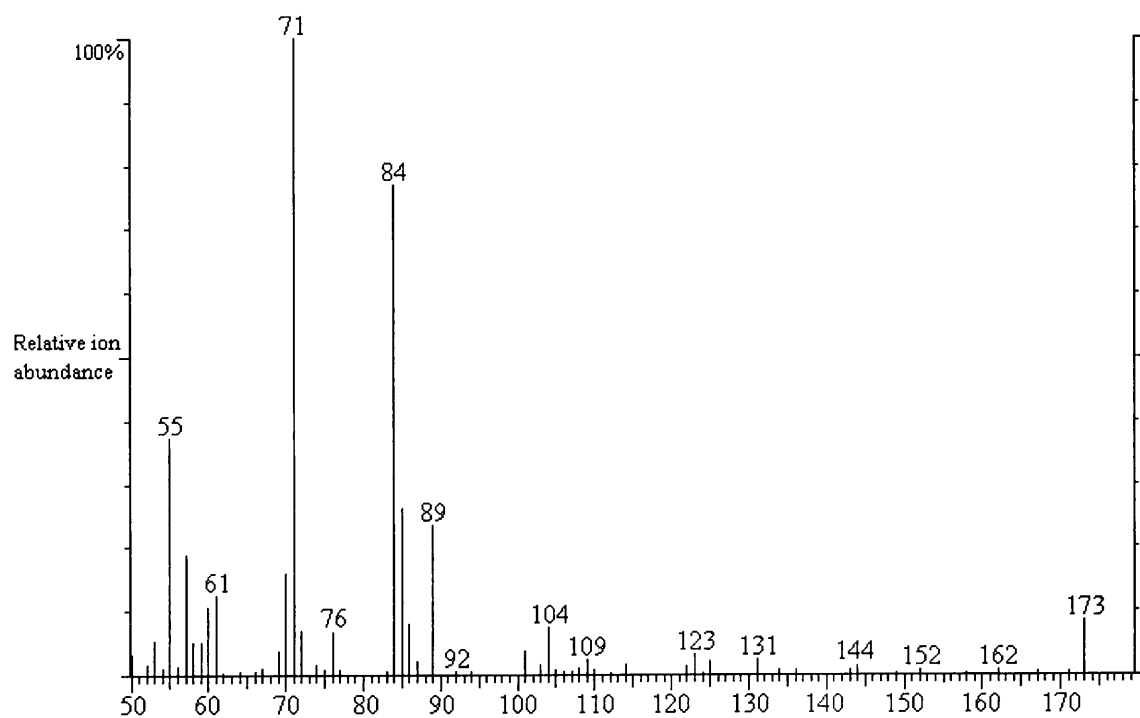
A2 MADD urine sample,
Propanoylcarnitine lactone (scan number 401, Figure 3.3), EI mode.



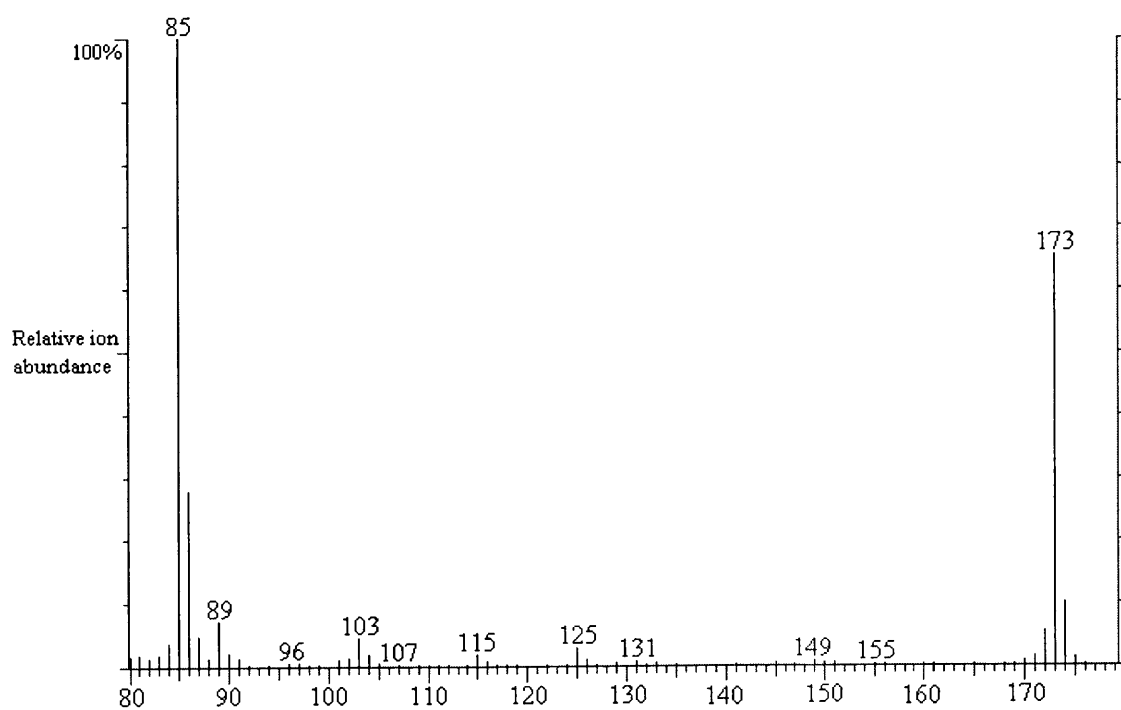
Propanoylcarnitine lactone, CI mode.



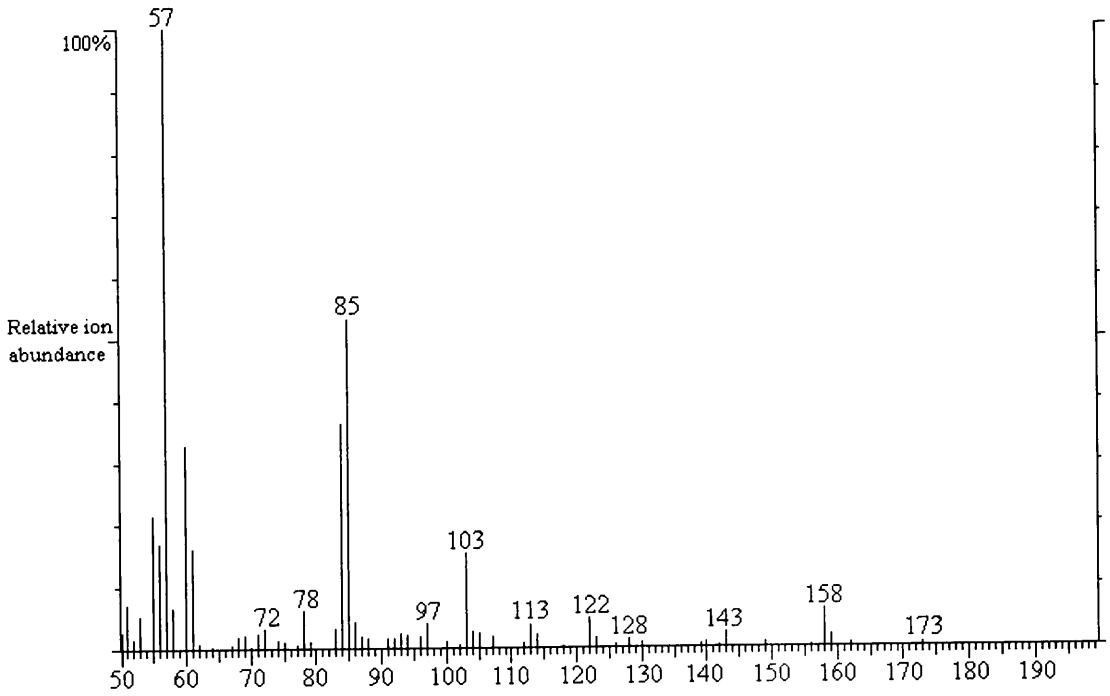
A3 MADD urine sample,
Isobutanoylcarnitine lactone (scan number 430, Figure 3.3), EI mode.



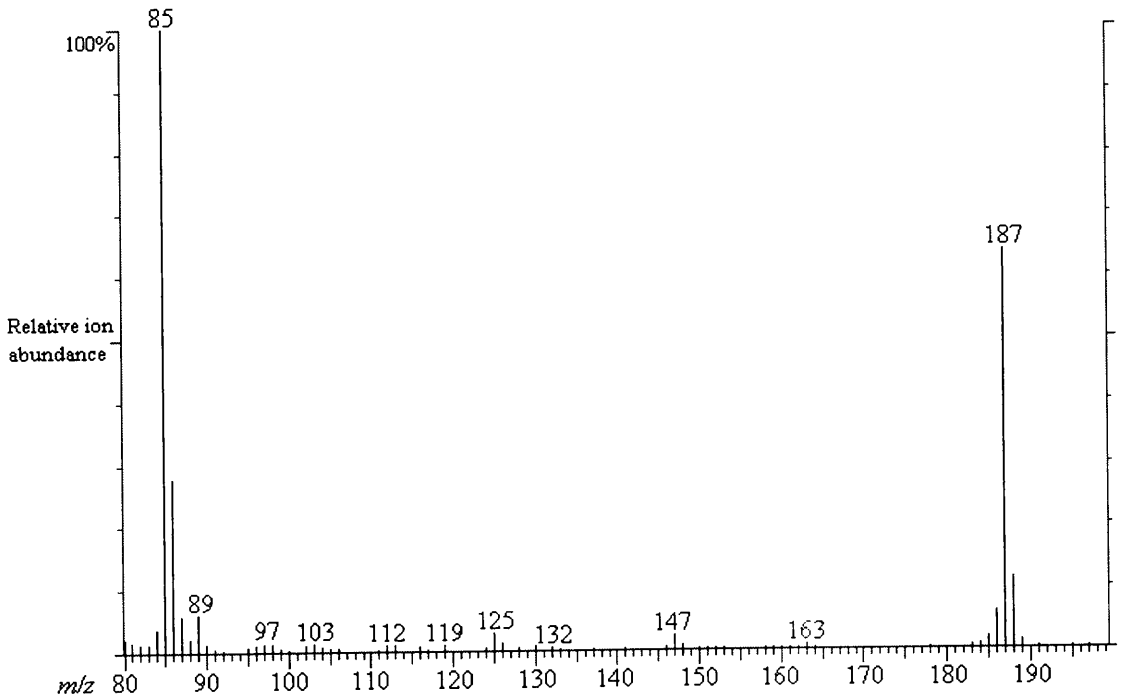
Isobutanoylcarnitine lactone, CI mode.



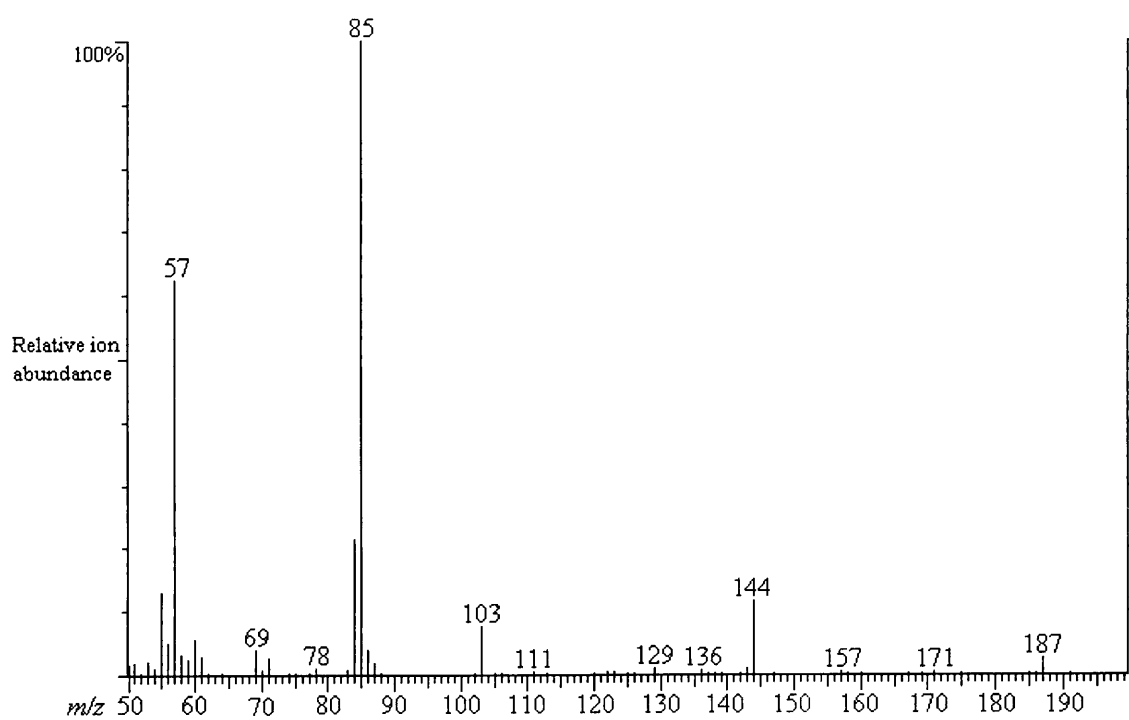
A4 MADD urine sample,
2-Methylbutanoylcarnitine lactone (scan number 503, Figure 3.3), EI mode.



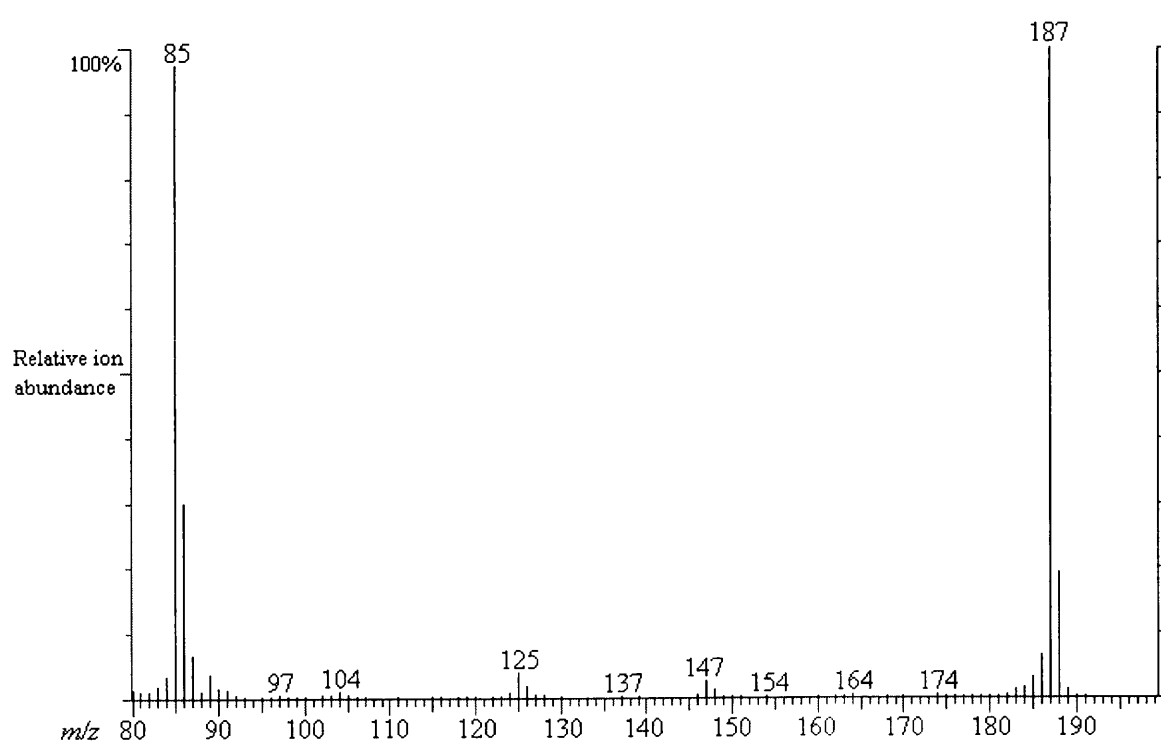
2-Methylbutanoylcarnitine lactone, CI mode.



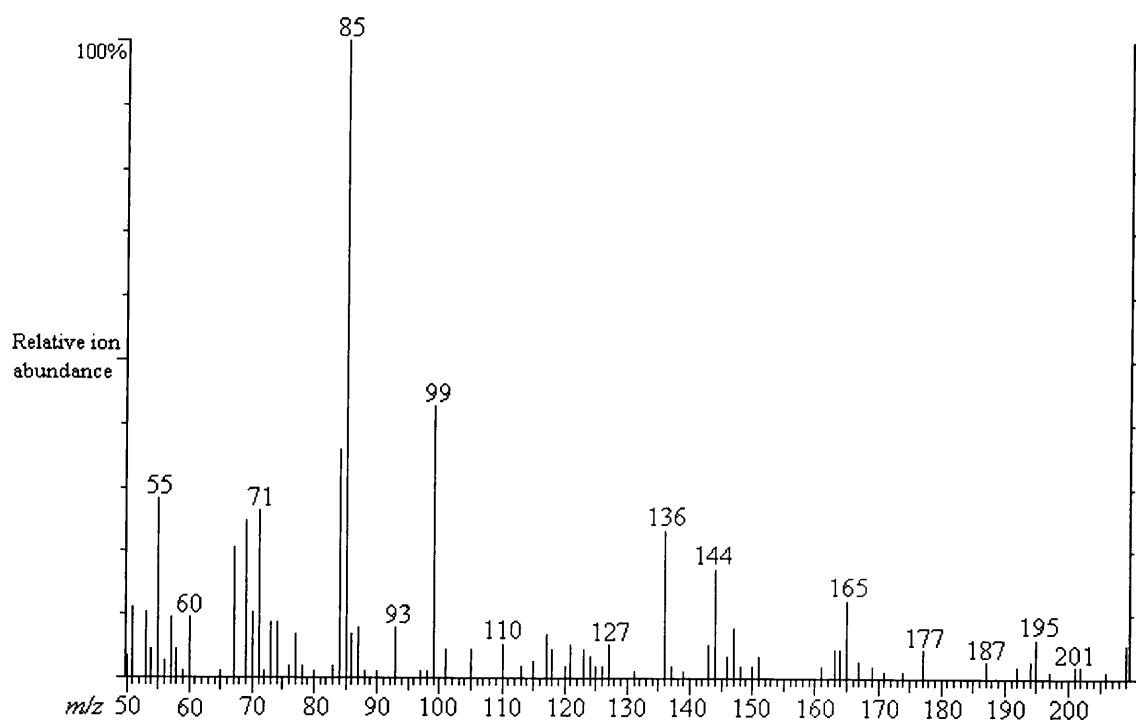
A5 MADD urine sample,
Isovalerylcarnitine lactone (scan number 511, Figure 3.3), EI mode.



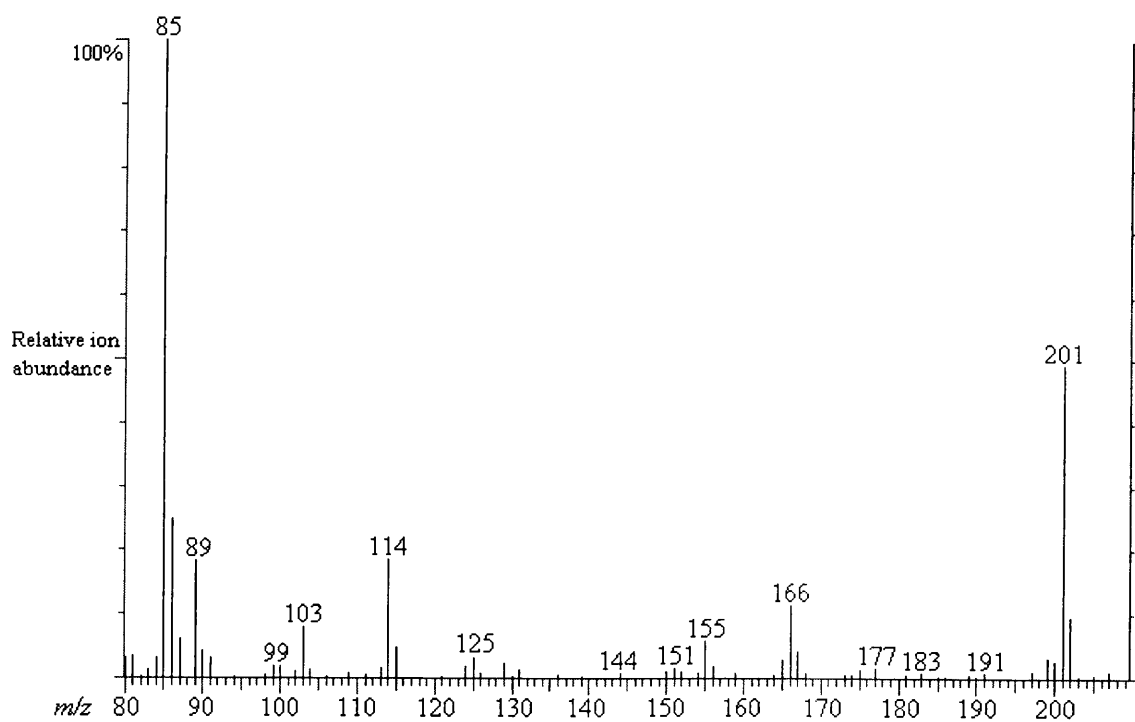
Isovalerylcarnitine lactone, CI mode.



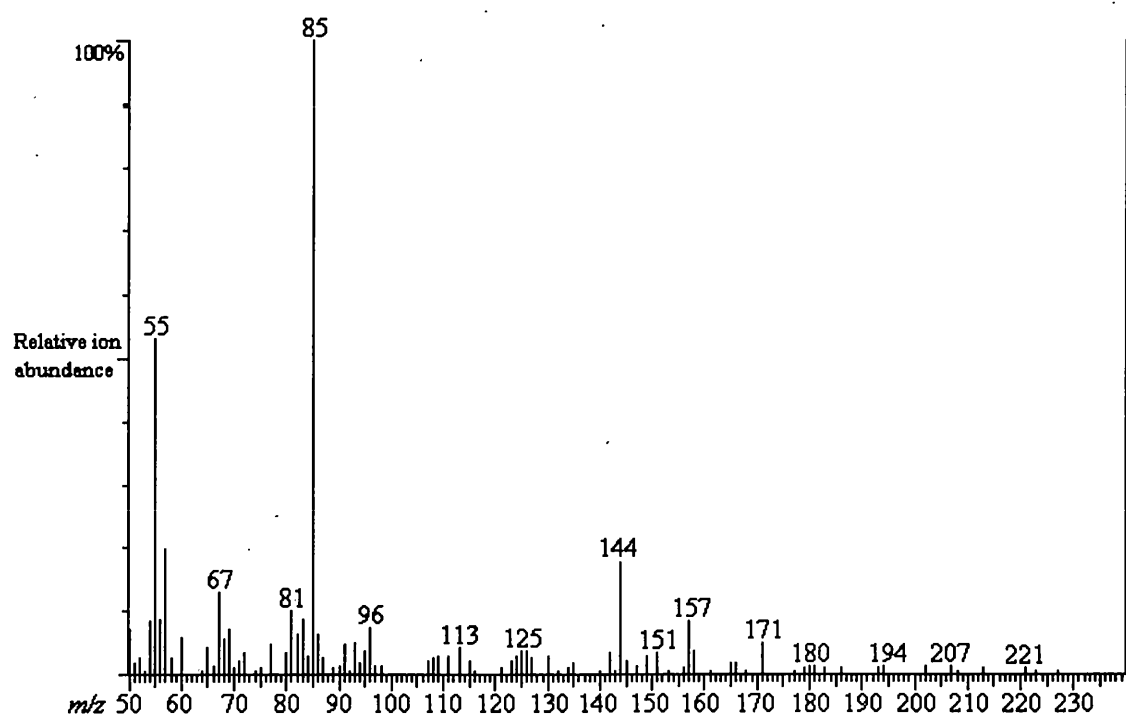
A6 MADD urine sample,
Hexanoylcarnitine lactone (scan number 626, Figure 3.3), EI mode.



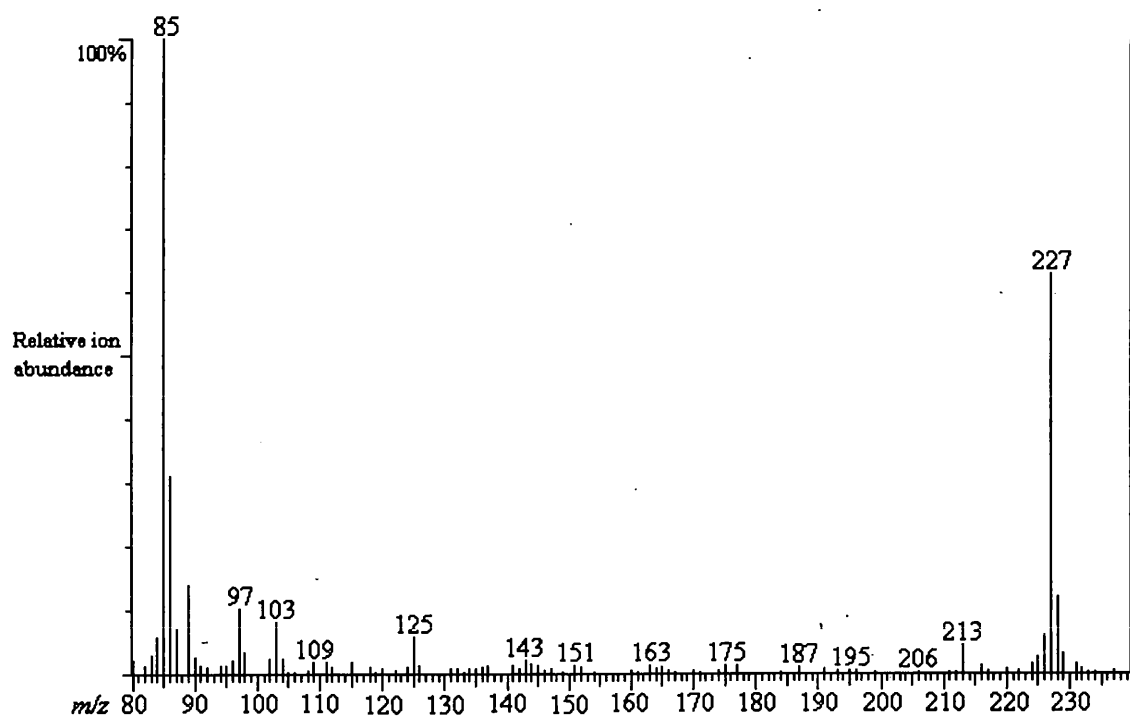
Hexanoylcarnitine lactone, CI mode,



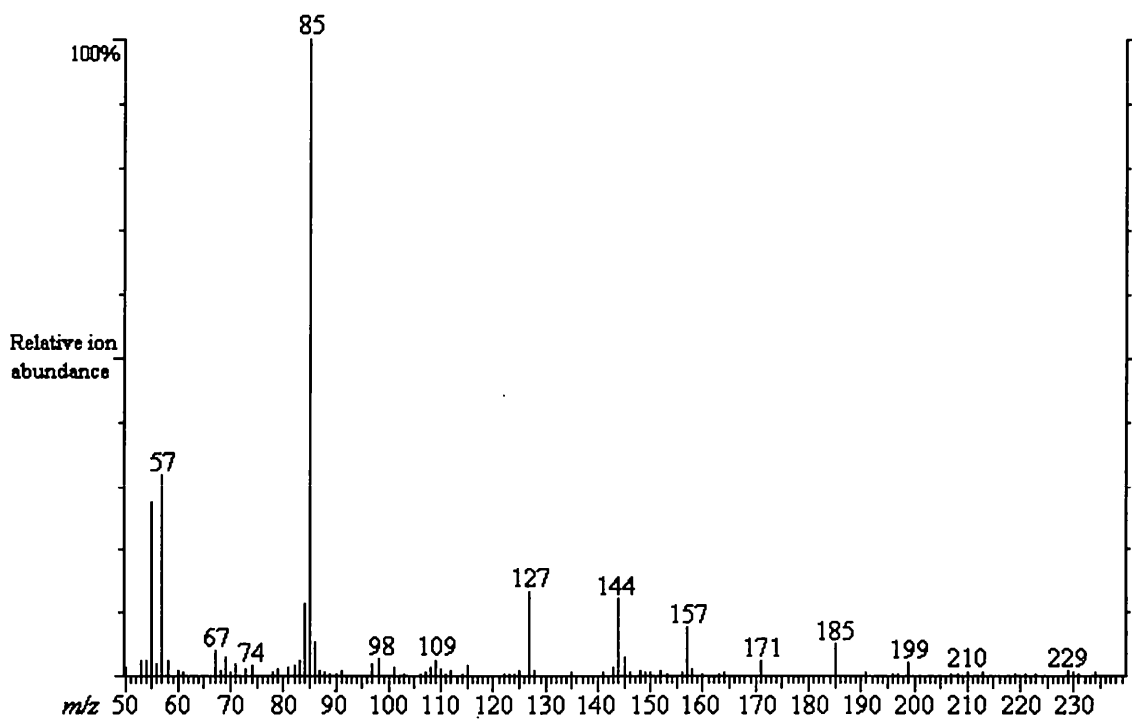
A7 MADD urine sample,
An octenoylcarnitine lactone (scan number 745, Figure 3.3), EI mode.



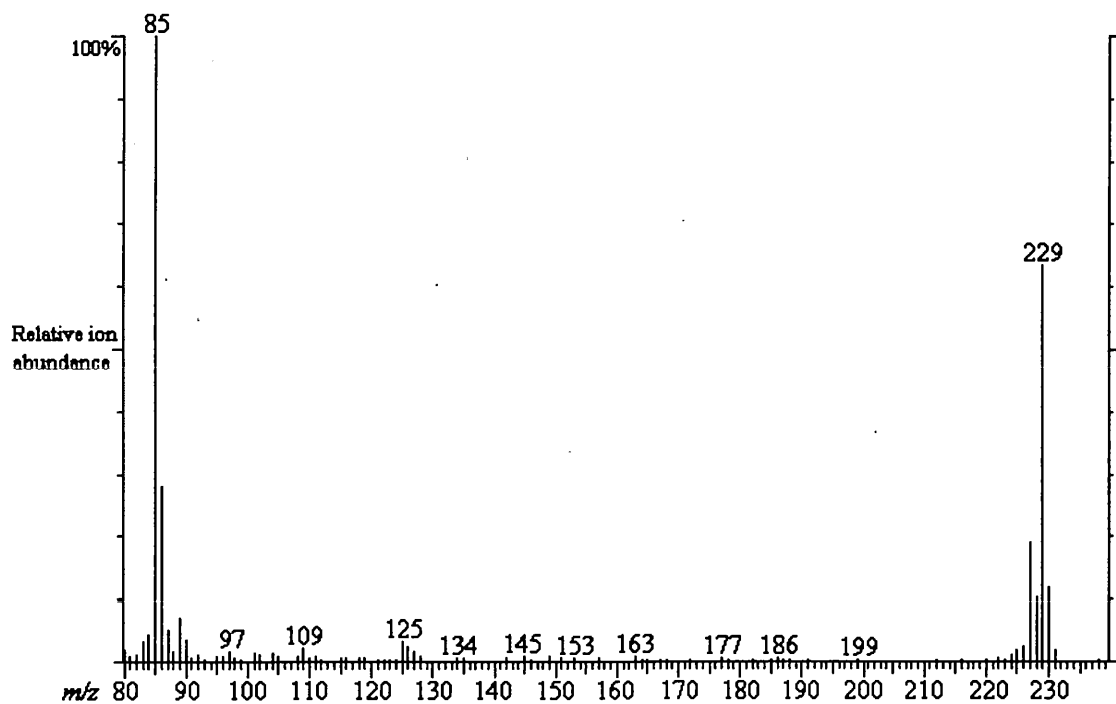
An octenoylcarnitine lactone, CI mode.



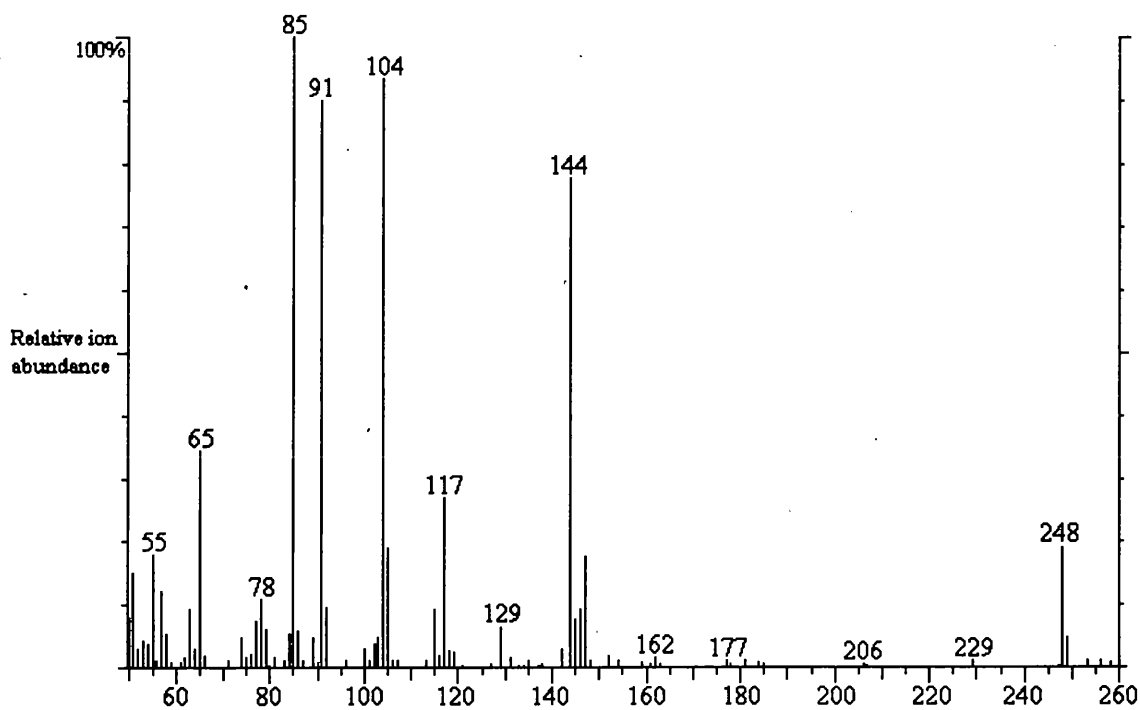
A8 MADD urine sample,
Octanoylcarnitine lactone (scan number 773, Figure 3.3), EI mode.



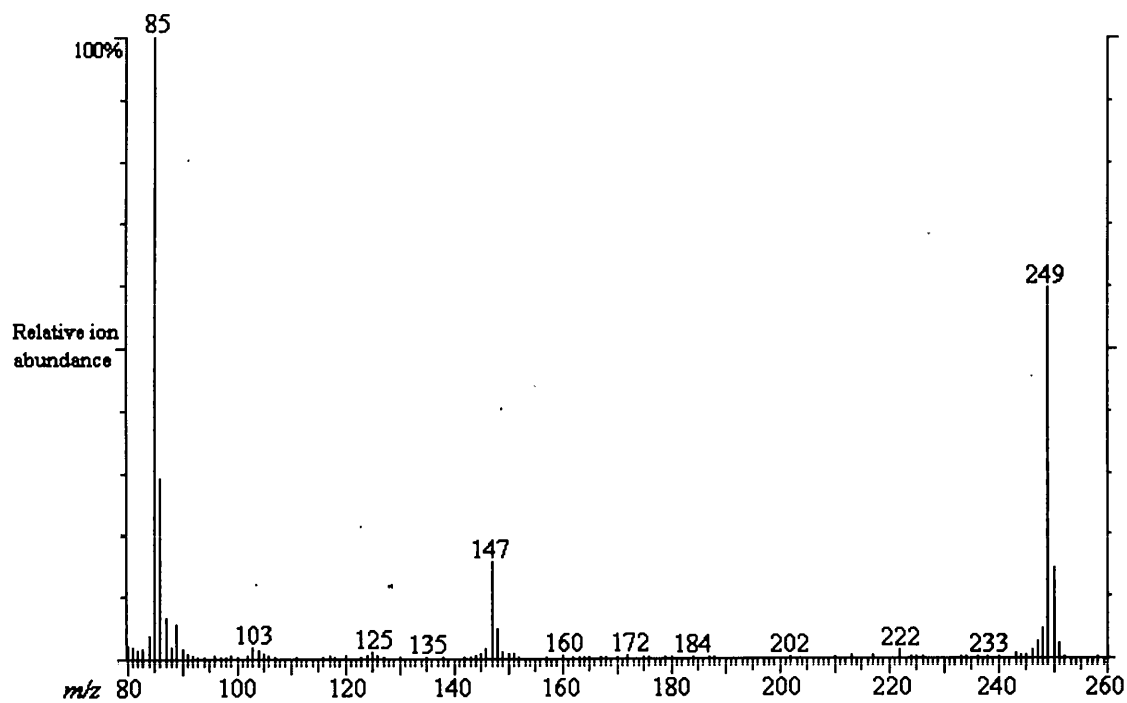
Octanoylcarnitine lactone, CI mode.



A9 MADD urine sample, (scan number 973, Figure 3.3)
4-Phenylbutanoylcarnitine lactone (internal standard), EI mode.



4-Phenylbutanoylcarnitine lactone (internal standard), CI mode,



APPENDIX B

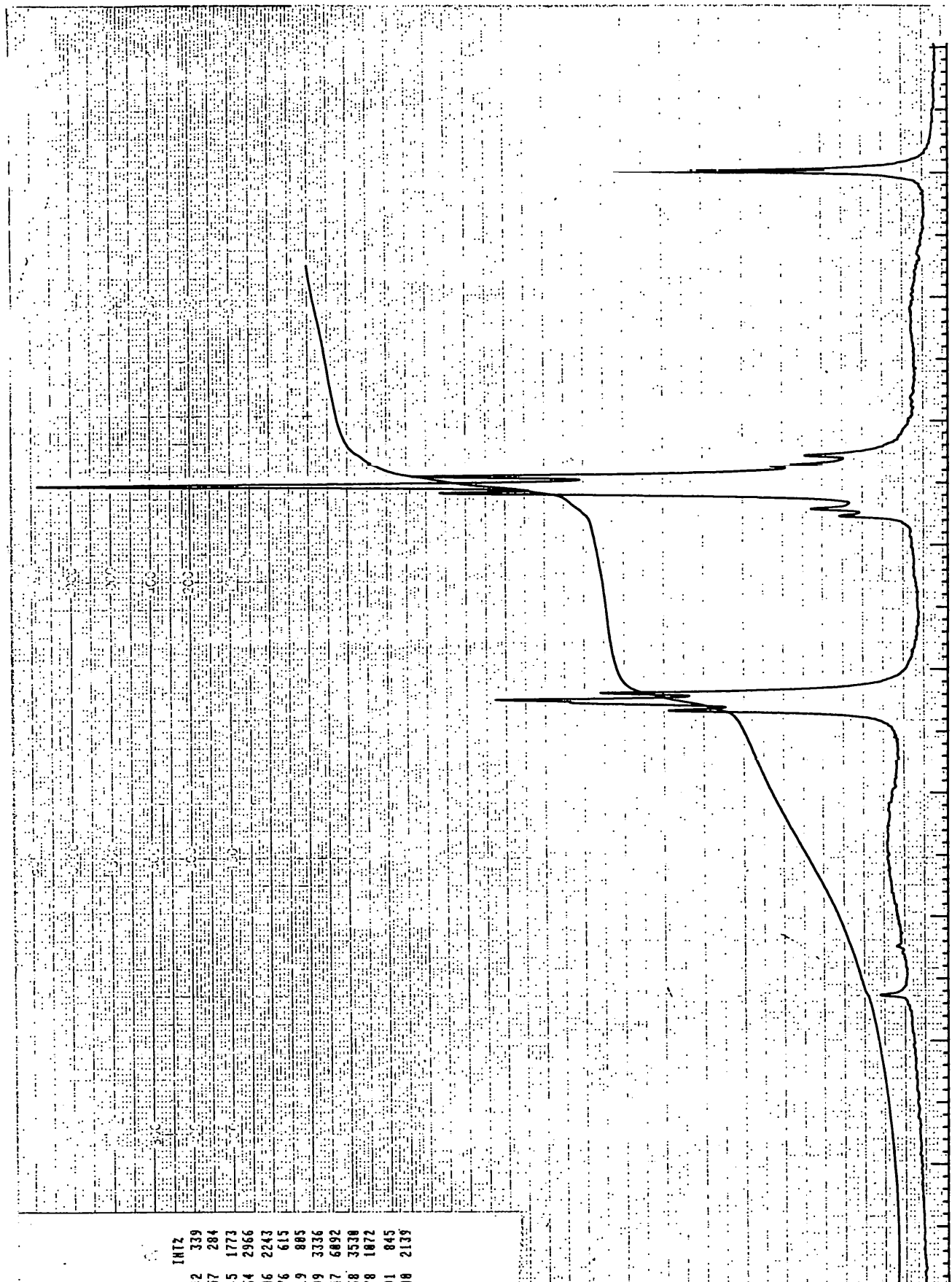
SUMMARY OF APPENDIX CONTENTS

- B1 ^1H NMR spectrum of Malic Acid
- B2 ^1H NMR spectrum of Cyclic Anhydride
- B3 Infrared spectrum of the β -hydroxy lactone
- B4 ^{13}C NMR spectrum of the β -hydroxy lactone
- B5 EI mass spectrum of isopropanol/diacid chloride reaction product
- B6 Infrared spectrum of Propanol/diacid chloride reaction product
- B7 Infra red spectrum of Cyclopentanol/diacid chloride reaction
- B8 Infrared spectrum of the Succinyl lactone
- B9 ^1H NMR spectrum of the Succinyl lactone
- B10 FAB spectrum of Succinyl lactone
- B11 Infrared spectrum of the Adipyl lactone
- B12 ^1H NMR and ^{13}C NMR spectra of the of Adipyl lactone
- B13 Positive and negative FAB spectra of the Adipyl lactone
- B14 ^{13}C NMR spectrum of the Succinylcarnitine
- B15 Positive FAB spectrum of Adipylcarnitine
- B16 ^1H NMR spectrum of Adipylcarnitine

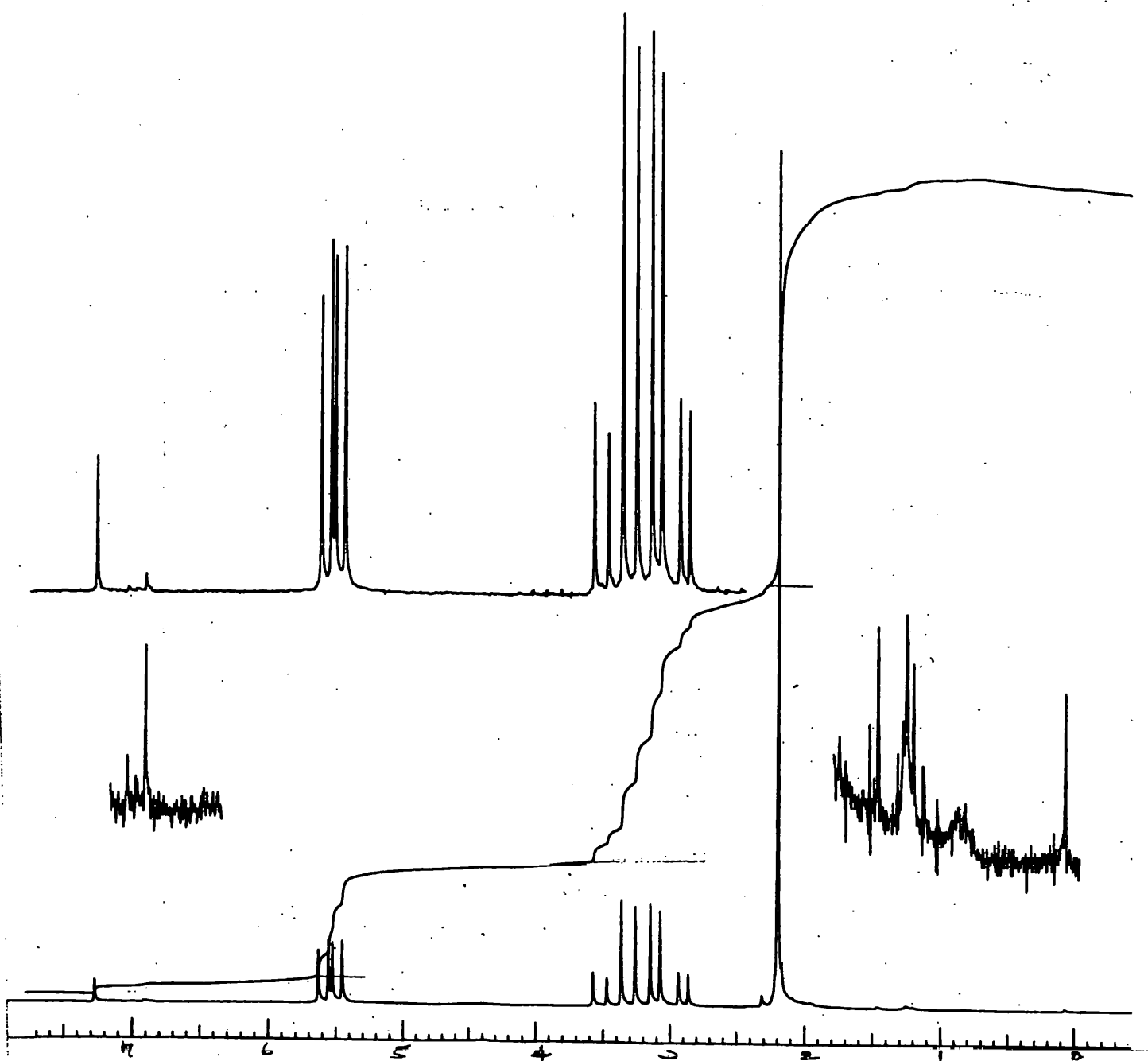
B1 ^1H NMR spectrum of Malic Acid

TOTAL 13
 RESOL24144 -5 HZ
 EXREF 0.000PPM
 OBS 407.9589 HZ
 NGAIN 9

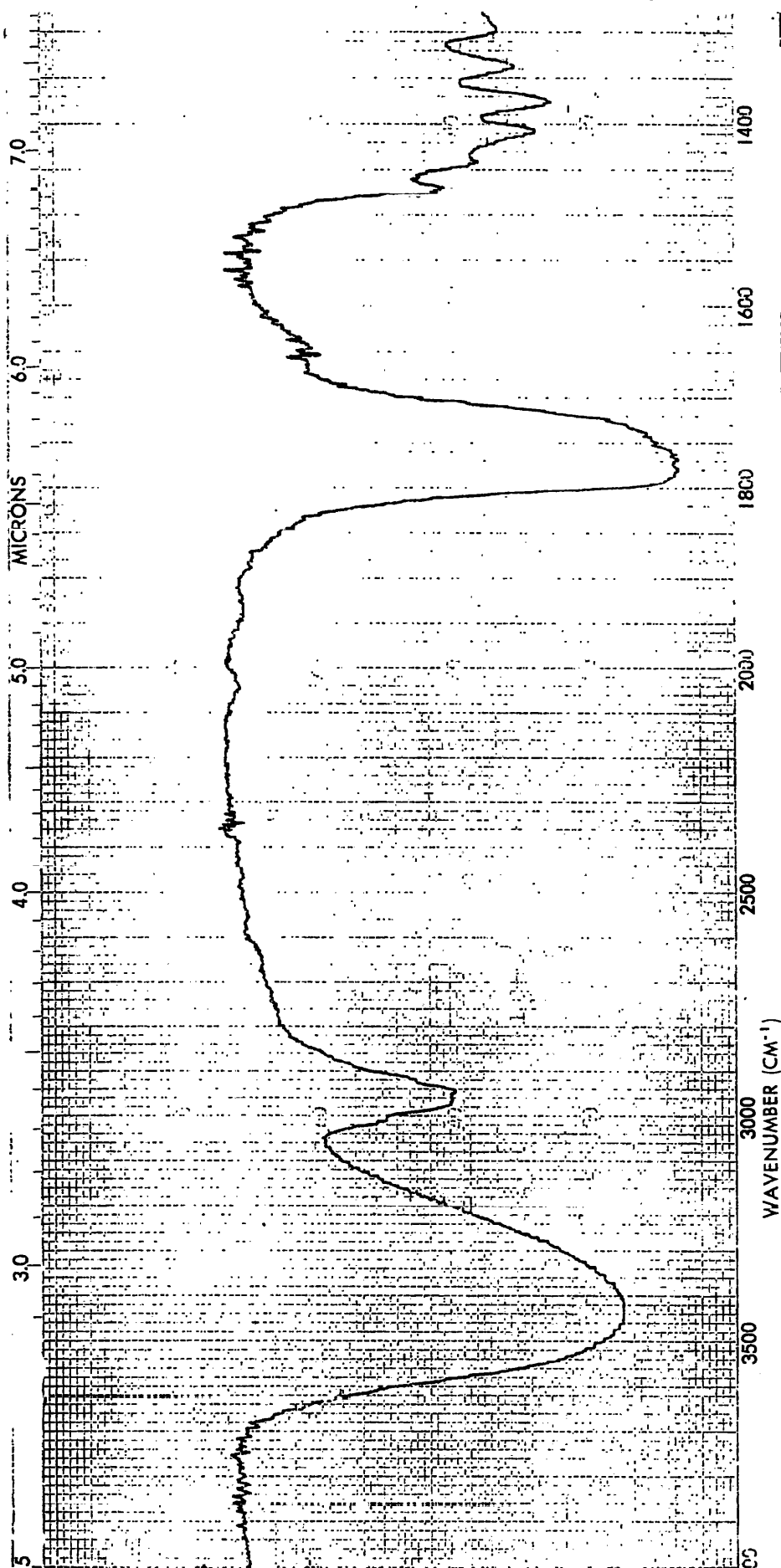
NO	FREQ(HZ)	PPM	INT2
1	595.21	6.642	339
2	489.01	5.457	284
3	389.40	4.345	1773
4	382.08	4.264	2966
5	376.95	4.206	2243
6	248.77	2.776	615
7	243.65	2.719	885
8	232.91	2.599	3336
9	228.27	2.547	6892
10	221.19	2.468	3538
11	213.13	2.378	1872
12	205.32	2.291	845
13	0.00	0.000	2139



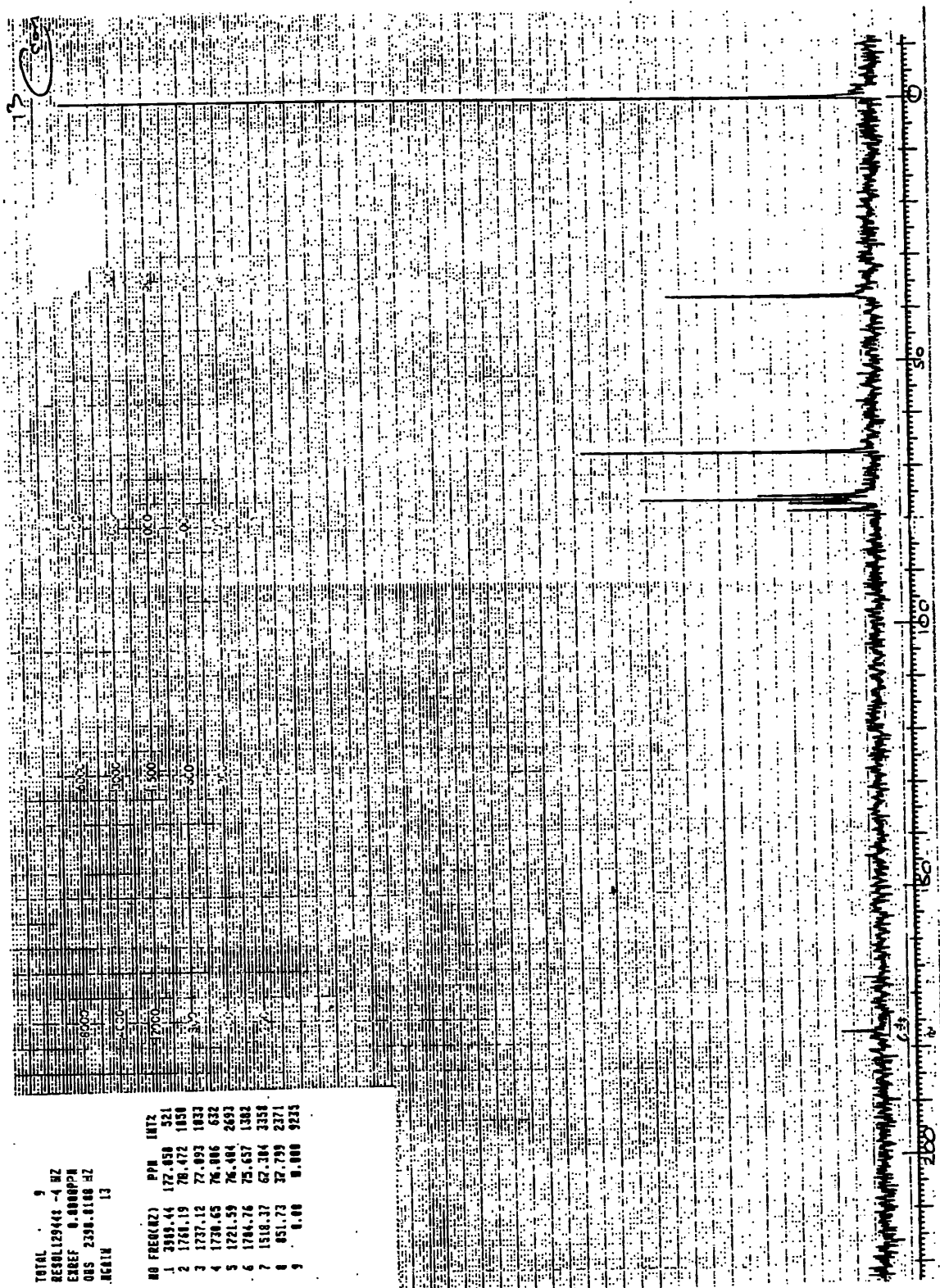
B2 ^1H NMR spectrum of Cyclic Anhydride



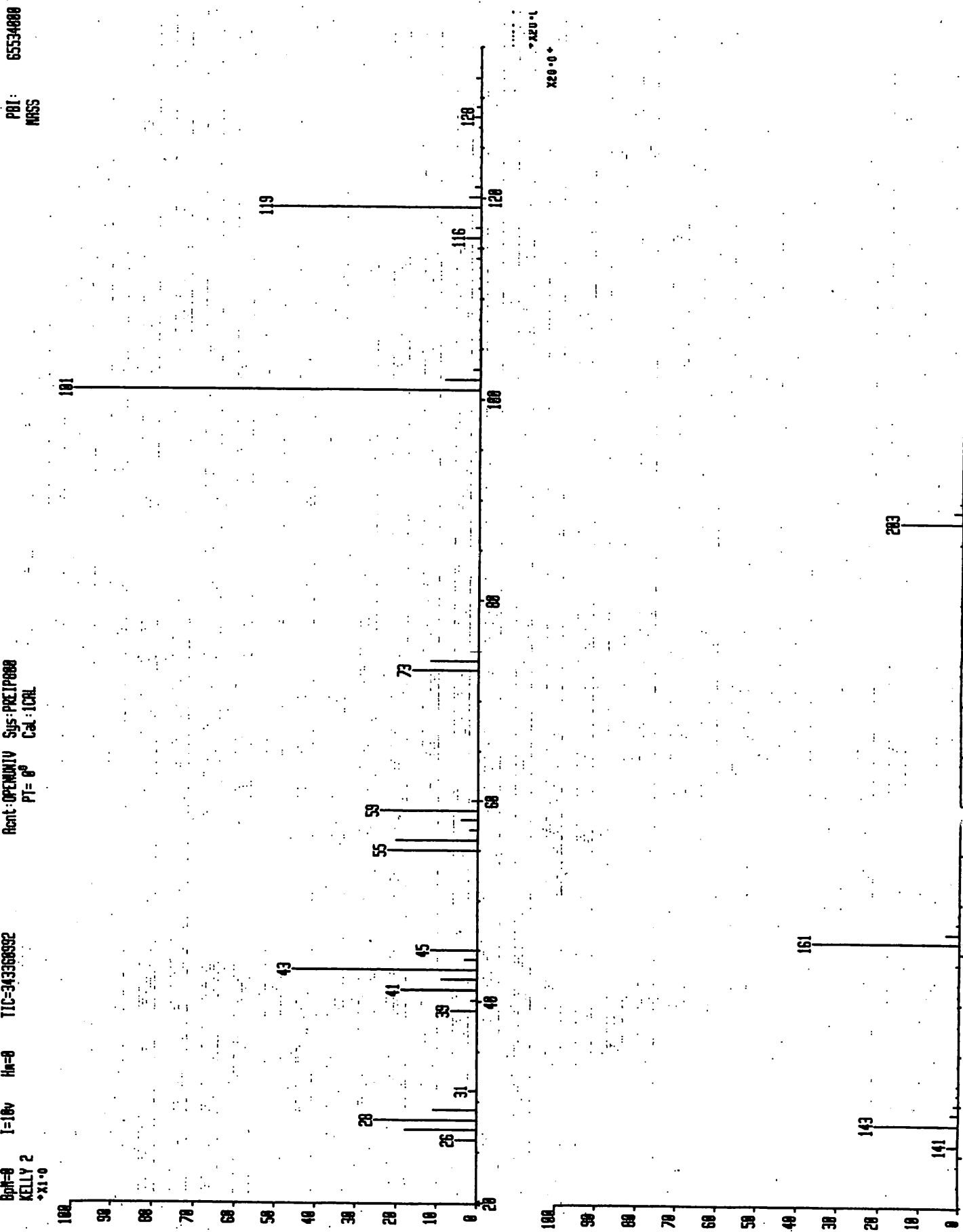
B3 Infrared spectrum of the β -hydroxy lactone

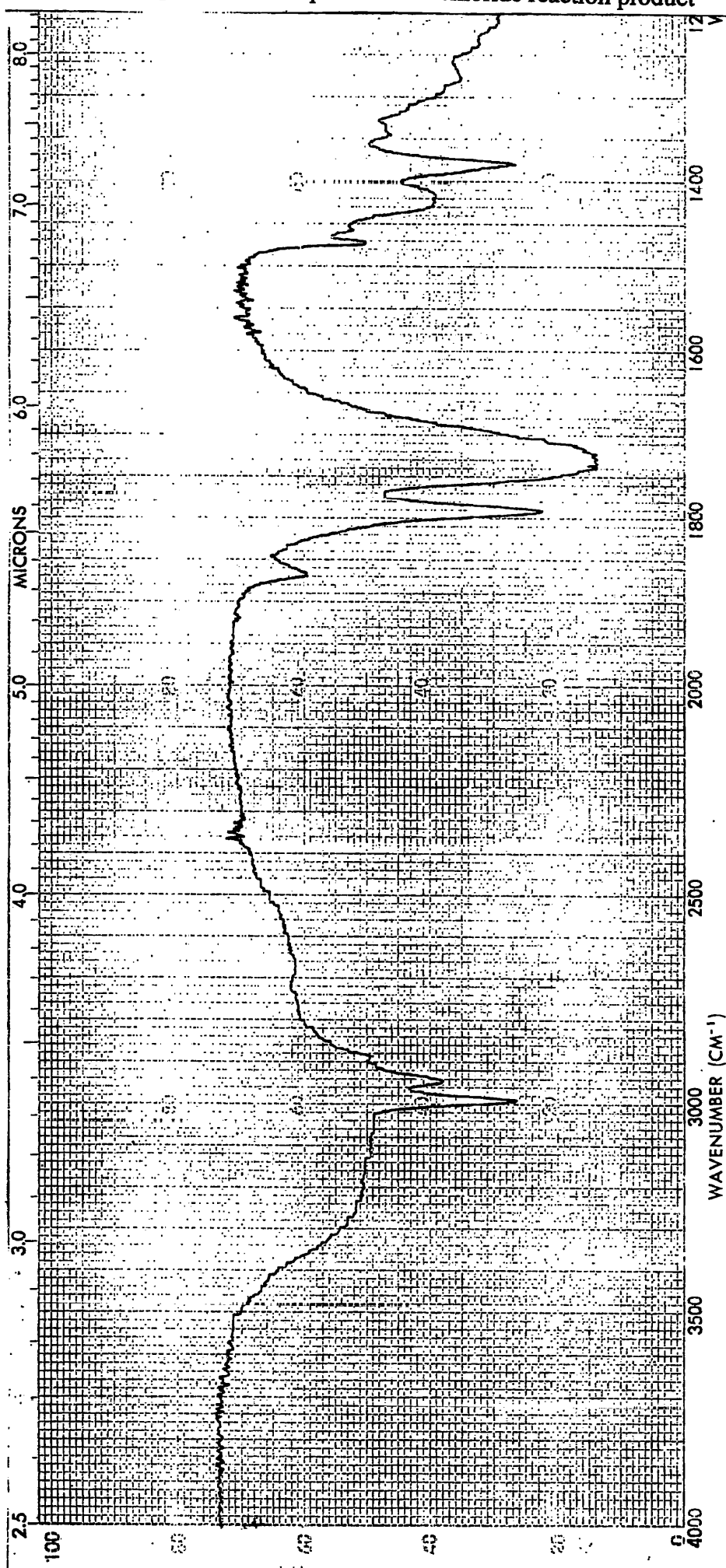


B4 ¹³C NMR spectrum of the β-hydroxy lactone

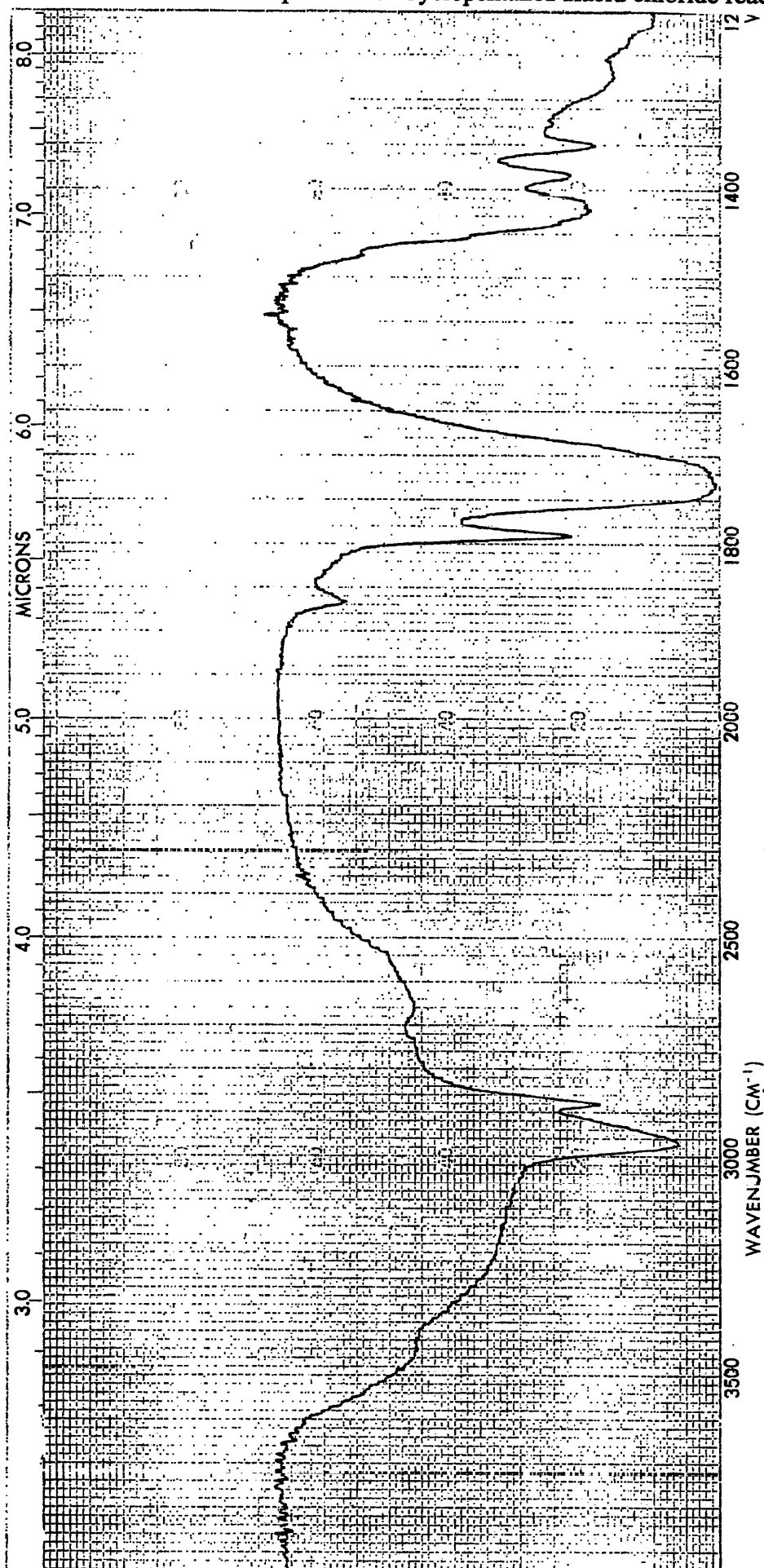


B5 EI mass spectrum of isopropanol/diacid chloride reaction product

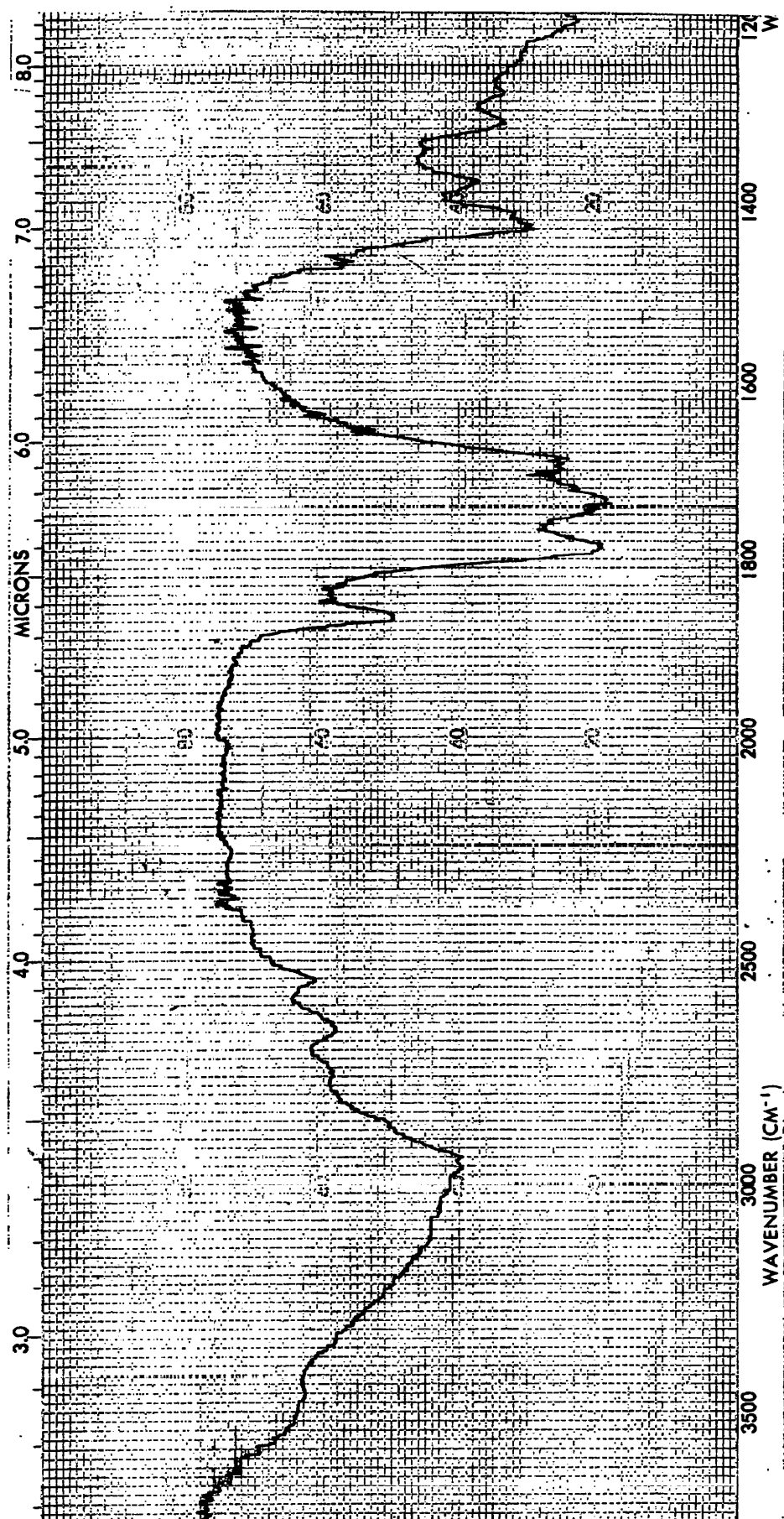


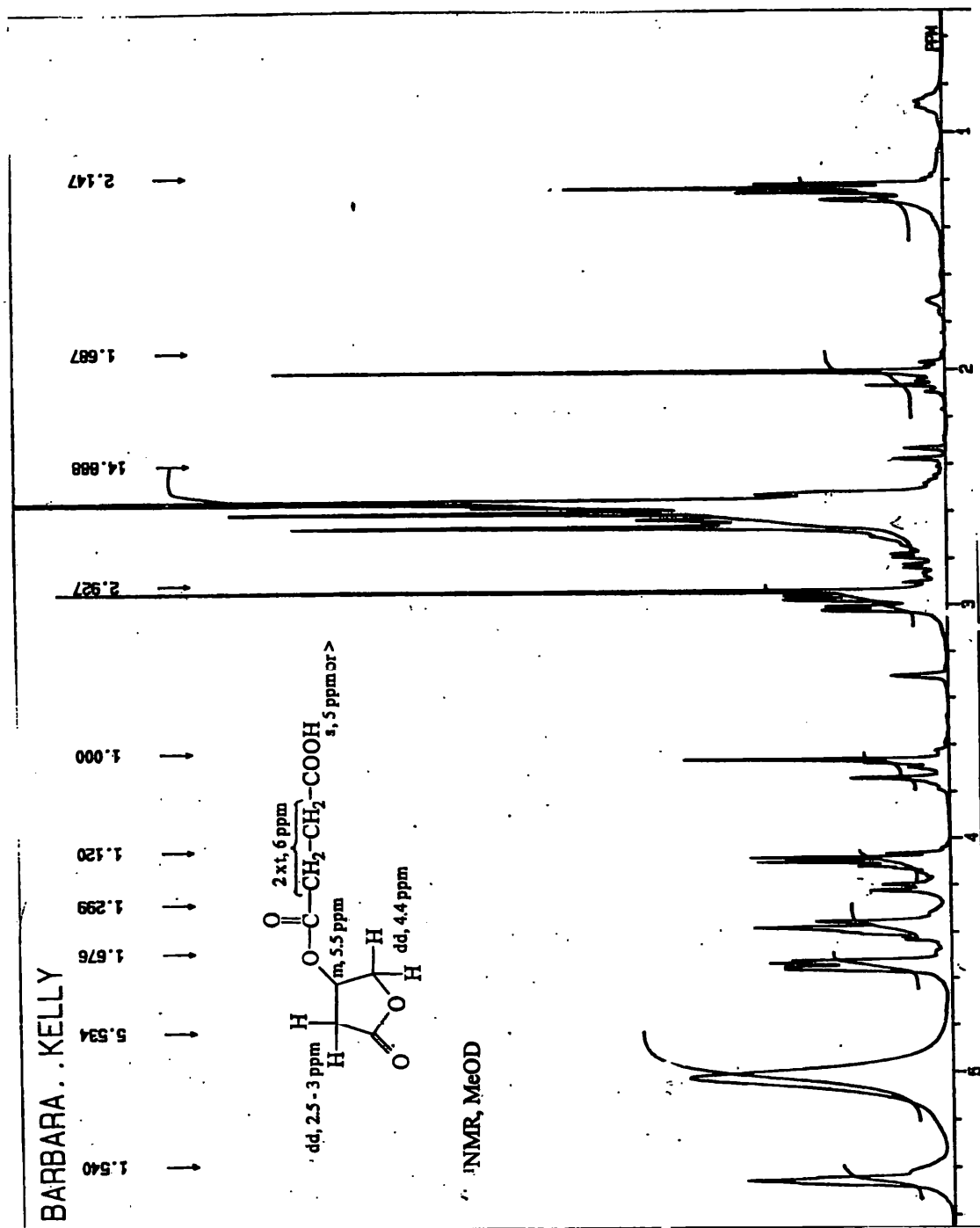


B7 Infrared spectrum of Cyclopentanol/diacid chloride reaction

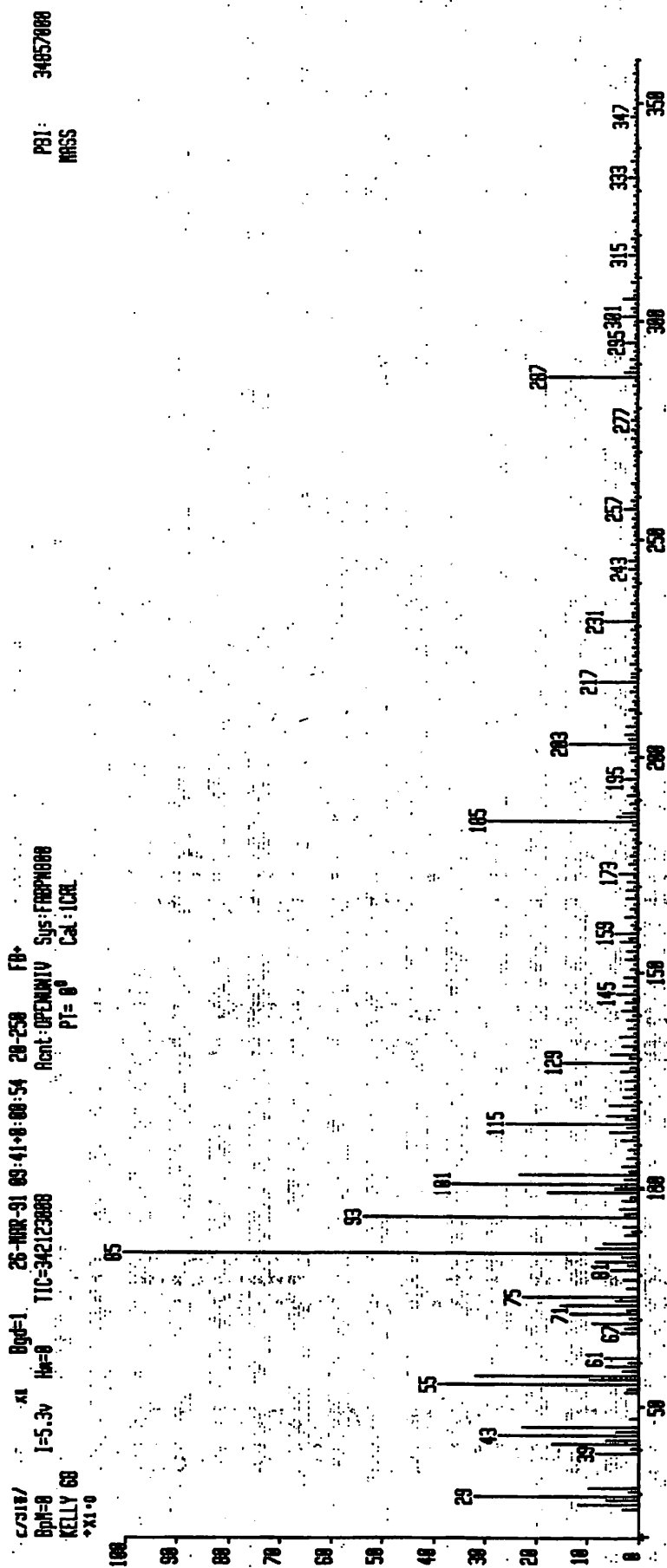


B8 Infrared spectrum of the Succinyl lactone

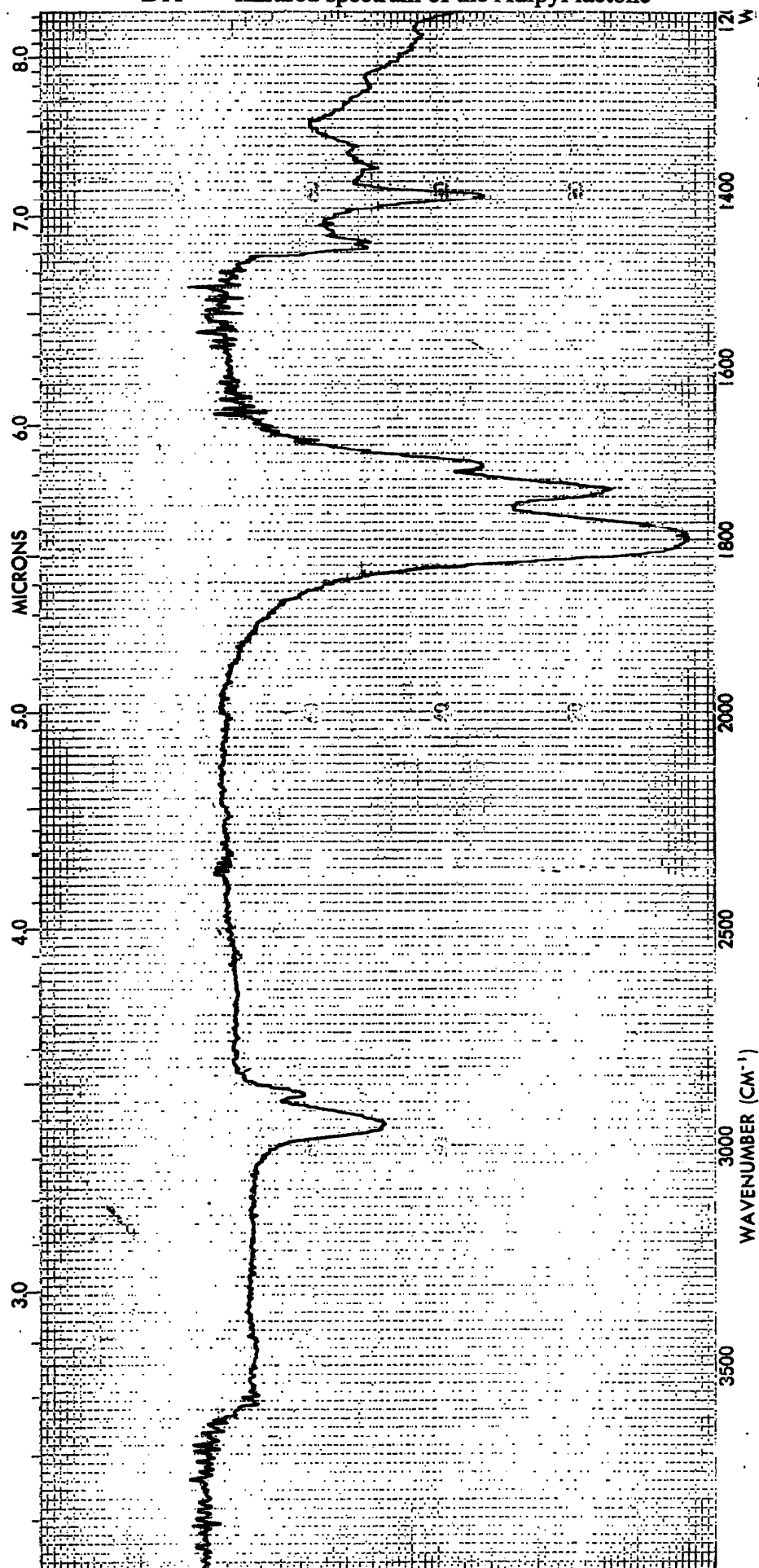


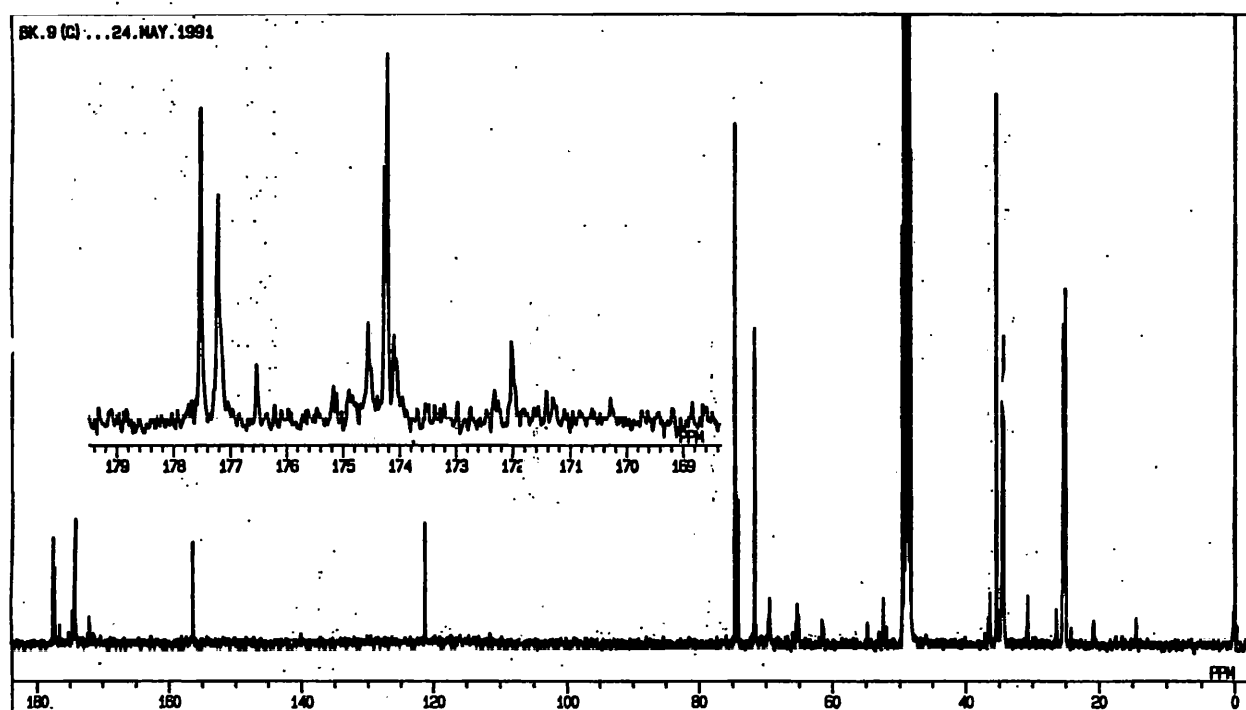
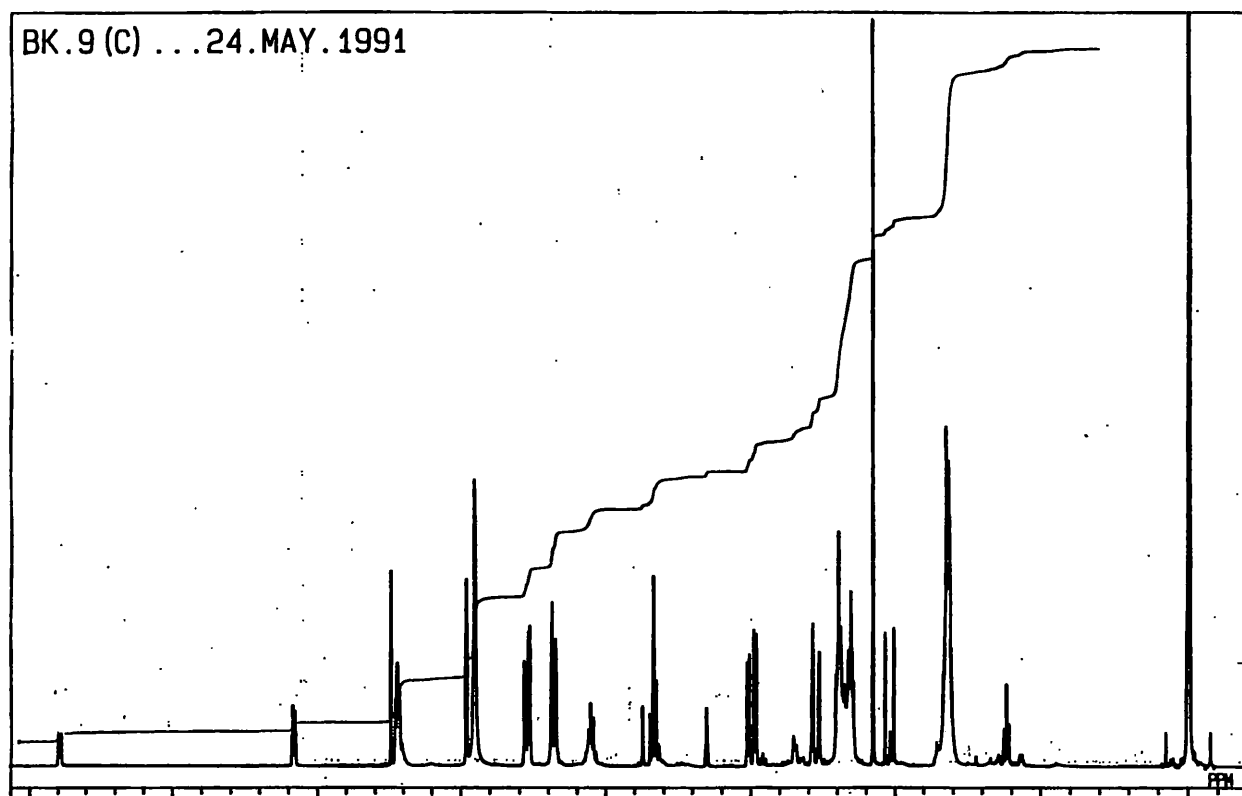


B10 FAB spectrum of Succinyl lactone



B11 Infrared spectrum of the Adipyl lactone

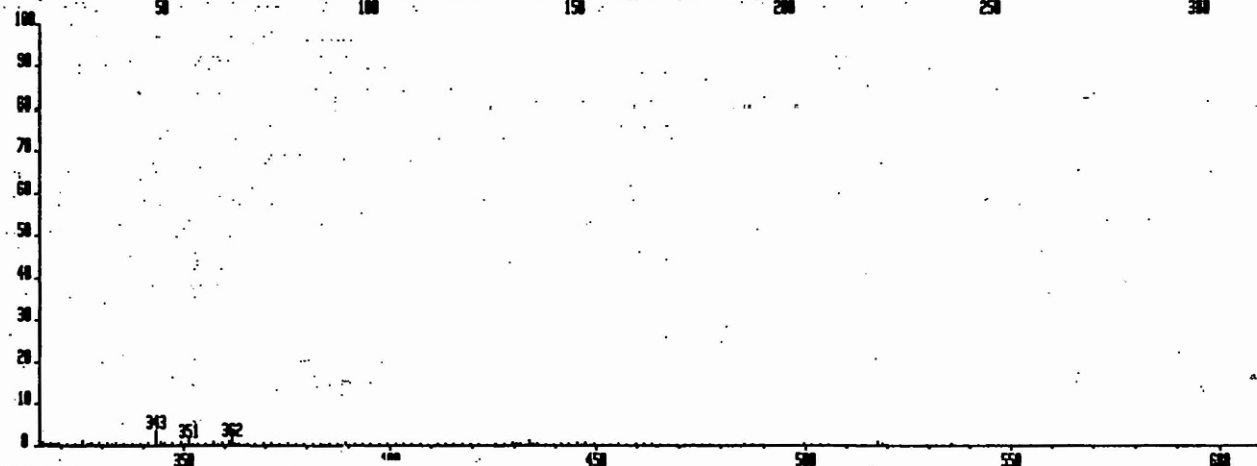
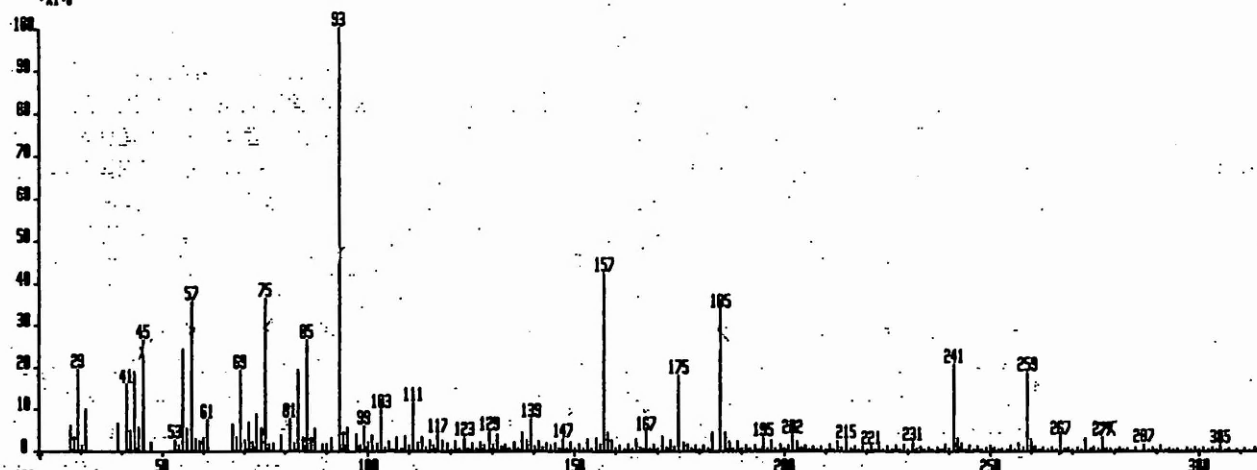




B13 Positive and negative FAB spectra of the Adipyl lactone

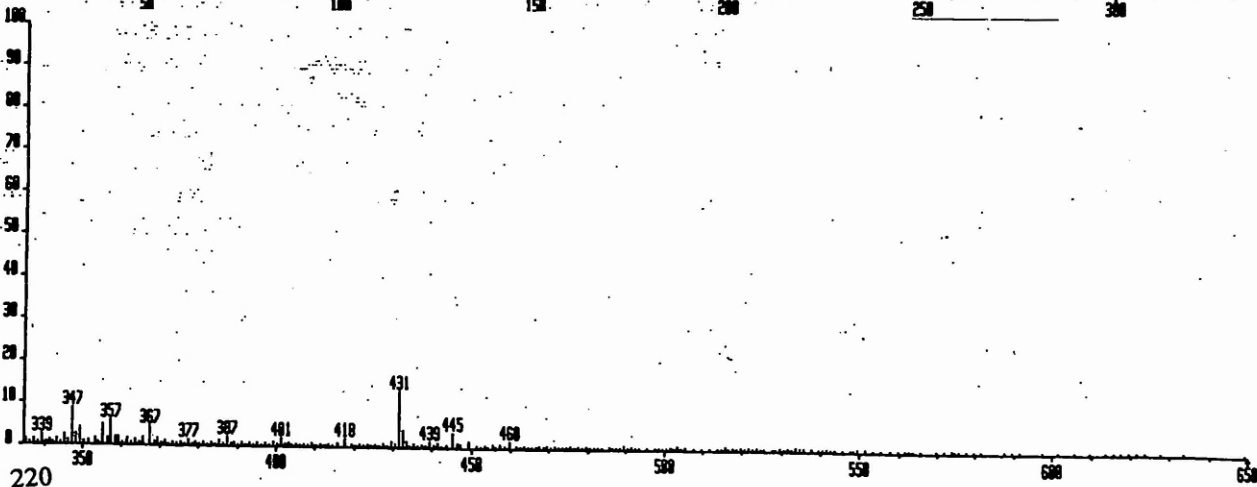
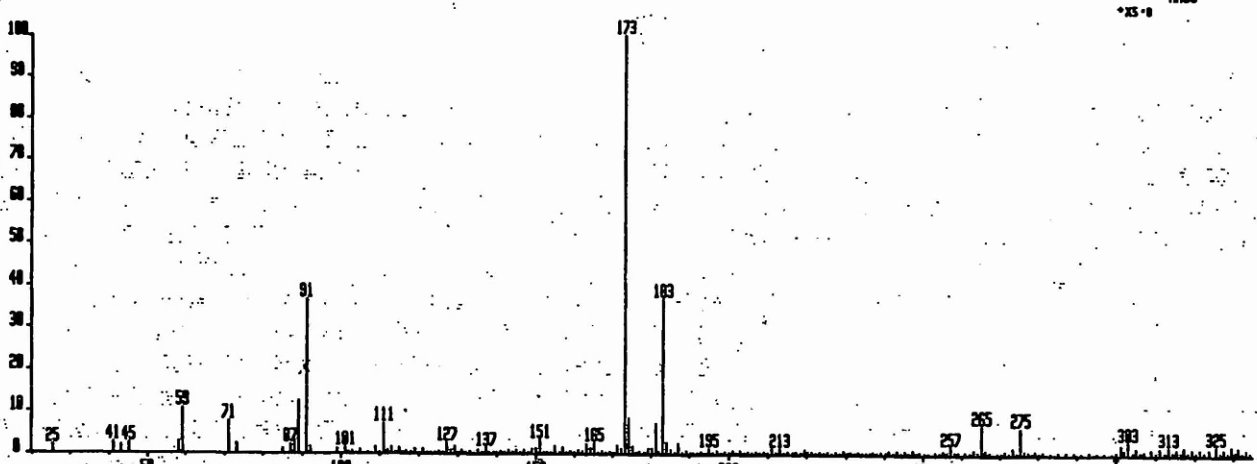
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 KELLY 76 PT= 0 Cal:ICOL

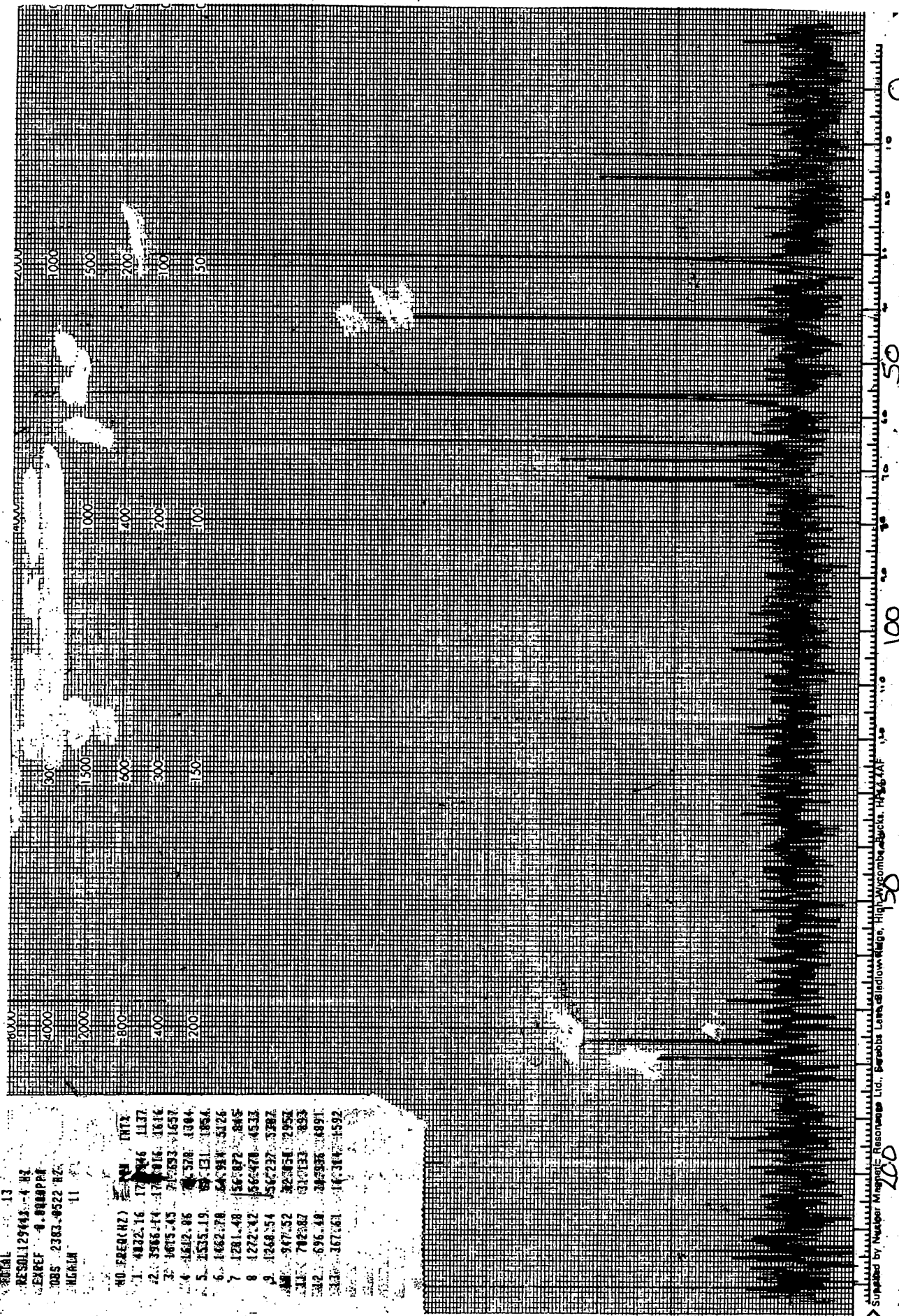
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 P055



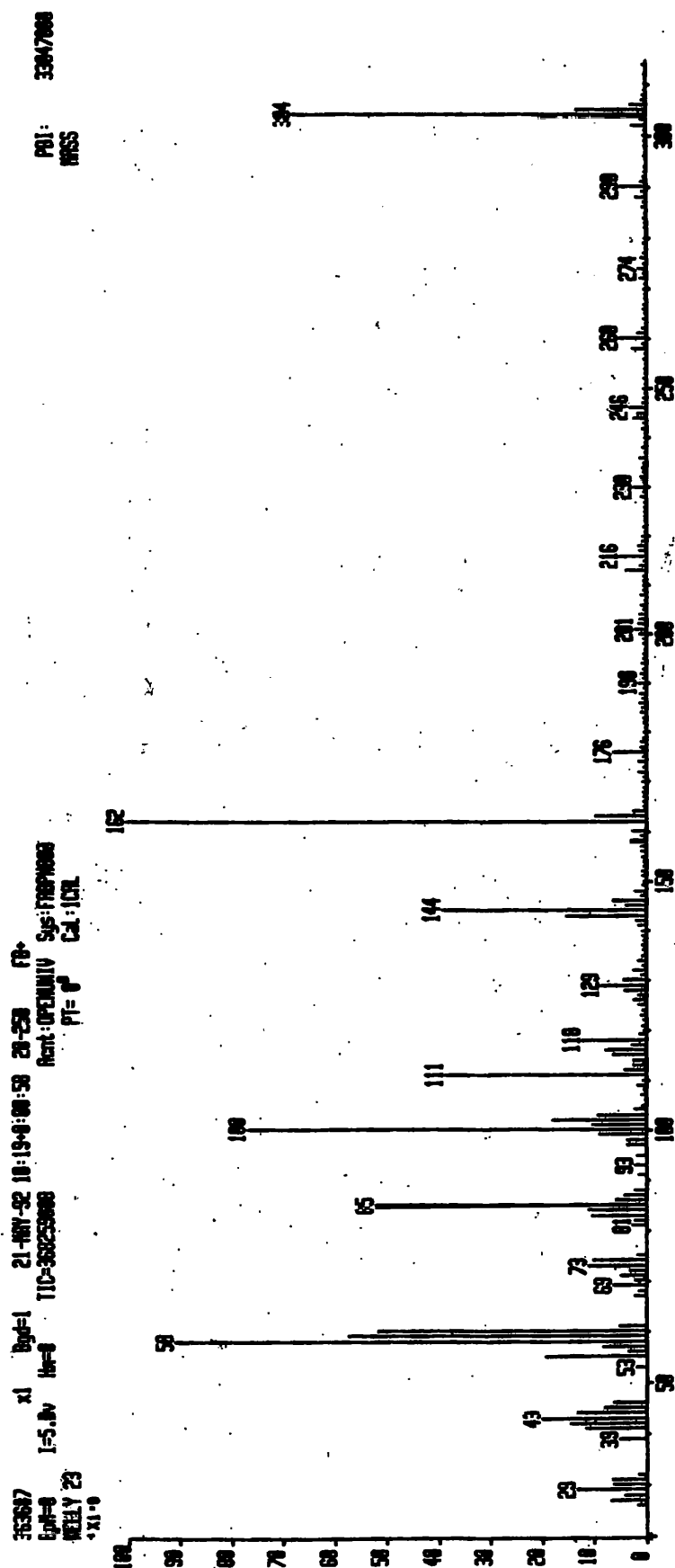
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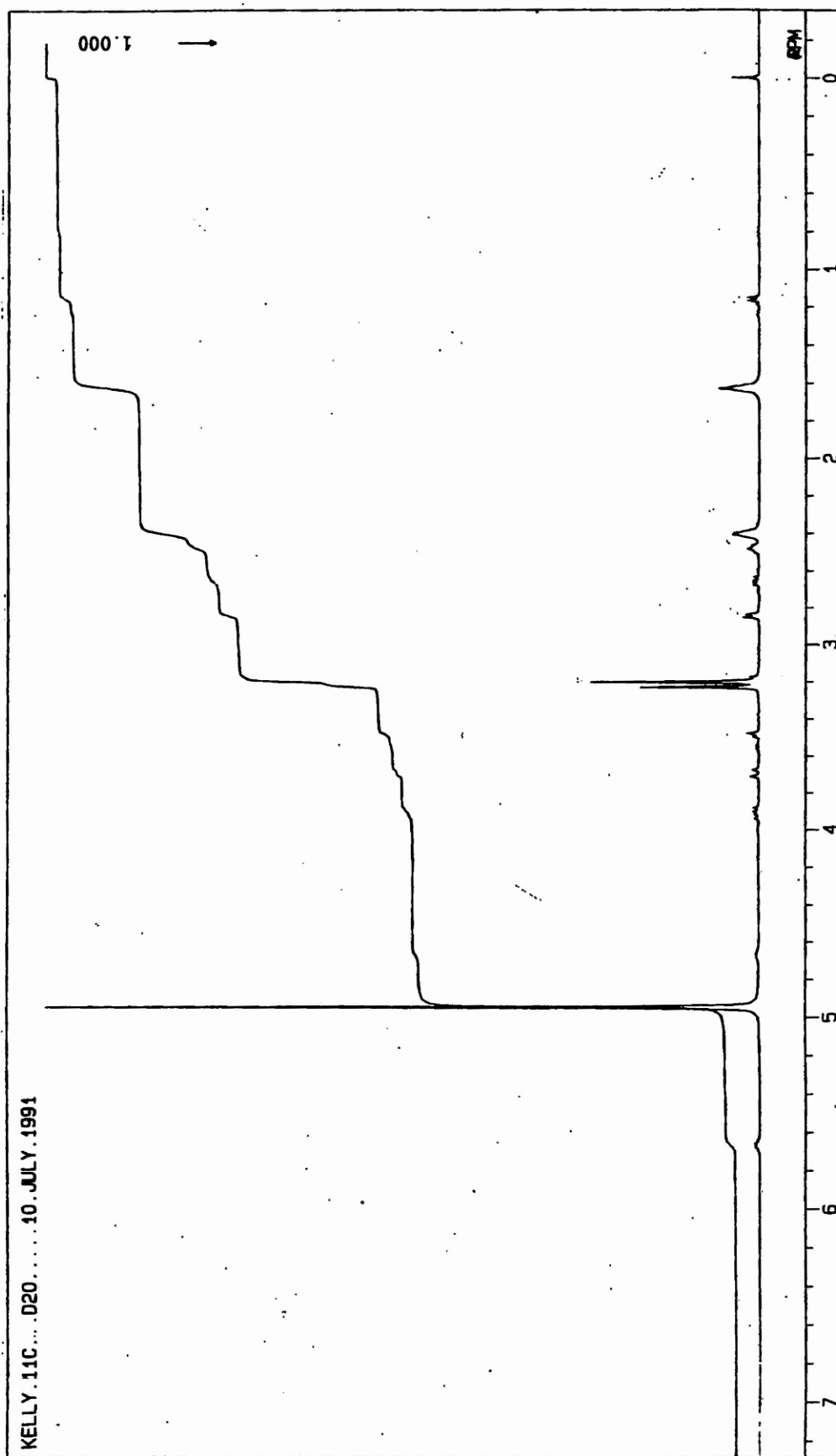




B15 Positive FAB spectrum of Adipylcarnitine



B16 ^1H NMR spectrum of Adipylcarnitine



APPENDIX C

GLOSSARY OF MEDICAL TERMS

Acidemia	A decrease in the pH of the blood.
Acidosis	A condition in which there is either (i) production by the body of two abnormal acids - β -hydroxybutyric or acetoacetic acids or (ii) a decrease
Apnoea	Stoppage of breathing, such as occurs when blood is artificially supplied with too much oxygen (i.e. taking several deep breaths in quick succession).
Arrhythmia	Variation from the normal rhythm of heart beat.
Congenital	Conditions that are present at, and usually before, birth regardless of the source of the disorder.
Epileptic	Paroxysmal transient disturbances of brain function which may be manifested as episodic impairment or loss of consciousness, abnormal motor phenomena, physis or sensory disturbances or perturbations of the autonomic nervous system with symptoms due to disturbances of the electrical activity of the brain
Hyperammonaemia	Abnormally high levels of ammonia in the blood.
Hypoglycaemia	Abnormally diminished glucose content in the blood.

Hypotonia Diminished skeletal muscle tone (weakness and floppiness in babies).

Inborn Errors of Metabolism

A genetically determined biochemical disorder in which a specific enzyme defect produces a metabolic block that may have pathological consequences at birth.

Ischemia Deficiency of blood in part due to functional constriction or actual obstruction of a blood vessel.

Ketosis Abnormally elevated levels of ketone bodies in tissue and fluids.

Myopathy The wasting of certain muscles without any previous increase in muscles. Post-mortem investigations have shown that the wasted muscle fibres have to a great extent been replaced with fatty and fibrous tissue (also known as muscular dystrophy).

Riboflavin Heat stable factor of vitamin B complex (B_2), 6,7-dimethyl-9-[1'-D-ribityl]-isoaloxazin ($C_{17}H_{20}N_4O_6$).

Steatosis Fatty degeneration.

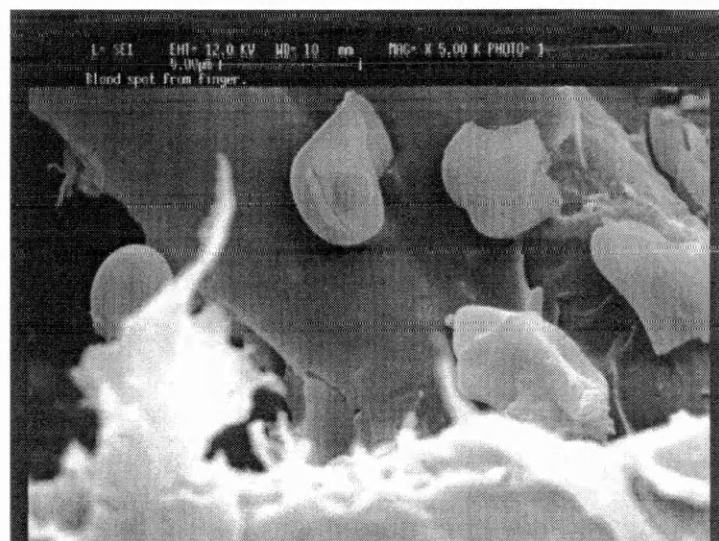
APPENDIX D

FATTY ACID PREFIXES/TRIVIAL NAMES FOR ACYLCARNITINES

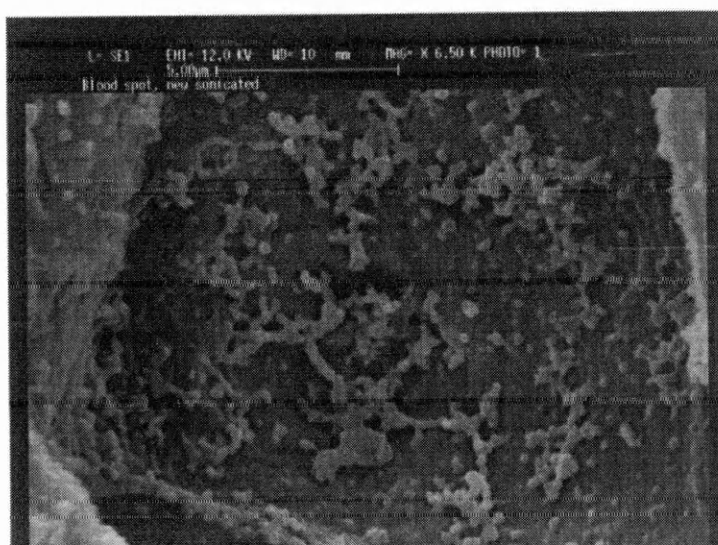
FATTY ACID PREFIXES FOR ACYLCARNITINES	TRIVIAL NAME(S)	STRUCTURE(S)
(A) MONOCARBOYLIC ACYLCARNITINES		
Acetylcarnitine		CH_3COO^-
Propanoyl	Propionyl	$\text{CH}_3\text{CH}_2\text{COO}^-$
Butanoyl		$\text{CH}_3(\text{CH}_2)_2\text{COO}^-$
Pentanoyl	Valeryl	$\text{CH}_3(\text{CH}_2)_3\text{COO}^-$
Hexanoyl	Caproyl	$\text{CH}_3(\text{CH}_2)_4\text{COO}^-$
Heptanoyl		$\text{CH}_3(\text{CH}_2)_5\text{COO}^-$
Octanoyl	Caprylyl	$\text{CH}_3(\text{CH}_2)_6\text{COO}^-$
Decanoyl	Caprionyl	$\text{CH}_3(\text{CH}_2)_8\text{COO}^-$
Dodecanoyl	Lauroyl	$\text{CH}_3(\text{CH}_2)_{10}\text{COO}^-$
Tetradecanoyl	Myristoyl	$\text{CH}_3(\text{CH}_2)_{12}\text{COO}^-$
Hexadecanoyl	Palmitoyl	$\text{CH}_3(\text{CH}_2)_{14}\text{COO}^-$
Octadecanoyl	Stearyl	$\text{CH}_3(\text{CH}_2)_{16}\text{COO}^-$
(B) BRANCH-CHAINED MONOCARBOYLIC ACYLCARNITINES		
2-Methylbutanoyl		$\text{CH}_3\text{CH}_2\text{CH}(\text{CH}_3)\text{COO}^-$
3-Methylbutanoyl	Isovaleryl	$(\text{CH}_3)_2\text{CHCH}_2\text{COO}^-$
2-Methylbut-2-enoyl	Tiglyl	$\text{CH}_3\text{CHC}(\text{CH}_3)\text{COO}^-$
2-Propylpentanoyl	Valproyl	$\text{CH}_3(\text{CH}_2)\text{CH}(\text{CH}_2\text{CH}_2\text{CH}_3)\text{COO}^-$
(C) DICARBOXYLIC ACYLCARNITINES		
Butanedioyl	Succinyl	$\text{HO}_2\text{C}(\text{CH}_2)_2\text{COO}^-$
Pentanedioyl	Glutaryl	$\text{HO}_2\text{C}(\text{CH}_2)_3\text{COO}^-$
Hexanedioyl	Adipyl	$\text{HO}_2\text{C}(\text{CH}_2)_4\text{COO}^-$
Octanedioyl	Suberyl	$\text{HO}_2\text{C}(\text{CH}_2)_6\text{COO}^-$
Decanedioyl	Subacyl	$\text{HO}_2\text{C}(\text{CH}_2)_8\text{COO}^-$
(D) BRANCH-CHAINED DICARBOXYLIC ACYLCARNITINES		
2-Methylpropandioyl	Methylmalonyl	$\text{HO}_2\text{CCH}(\text{CH}_3)\text{COO}^-$

APPENDIX E

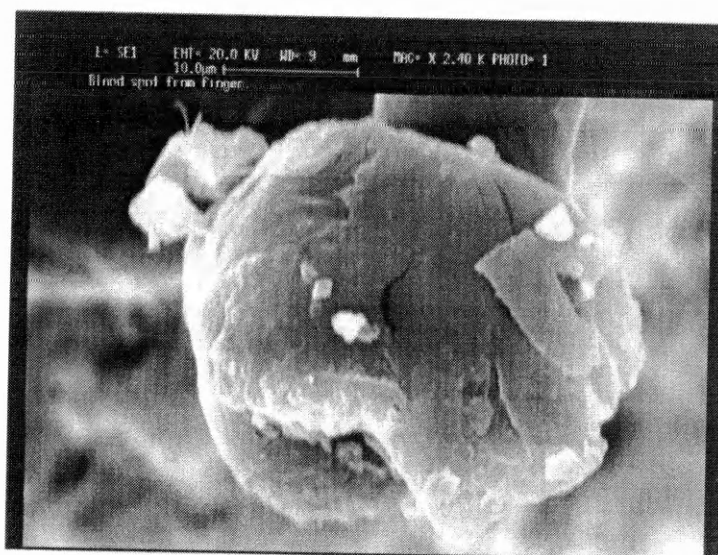
SCANNING ELECTRON MICROGRAPHS



E1 Fresh/untreated blood spot
x 5000 magnification



E2 Fresh blood spot, sonicated
x 6500 magnification



E3 White blood cell (20 – 30 μm)
x 2500 magnification



E4 Red blood cell (1 – 2 μm)
x 22,000 magnification



E5 Blood spot, 6 months after preparation
 x 8,000 magnification